(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
13 February 2003 (13.02.2003)

PCT

(10) International Publication Number
WO 03/011336 A2

(51) International Patent Classification[7]: A61K 39/39, 9/127, A61P 37/04, 35/00

(21) International Application Number: PCT/CA02/01217

(22) International Filing Date: 2 August 2002 (02.08.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/309,512 3 August 2001 (03.08.2001) US

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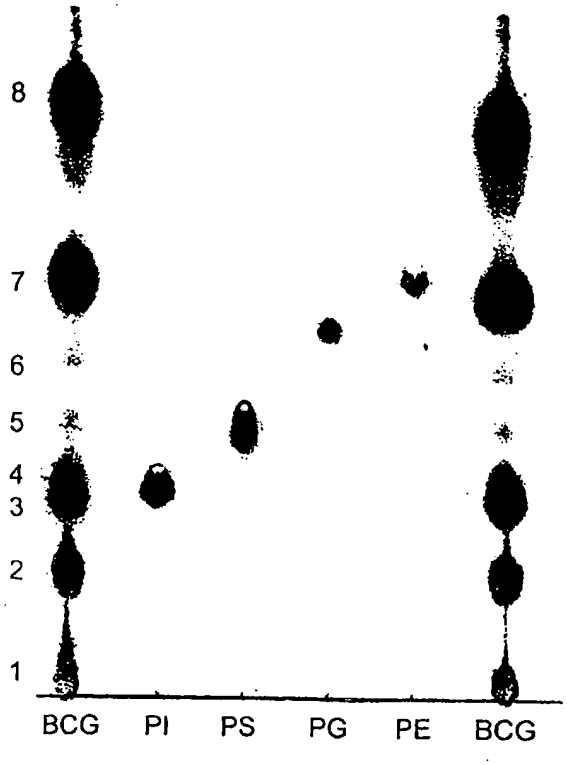
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),

*[Continued on next page]*

(54) Title: VACCINE ADJUVANT PROPERTIES OF LIPSOMES FORMED AT ELEVATED TEMPERATURES FROM THE POLAR CHLOROFORM EXTRACTABLE LIPIDS FROM *MYCOBACTERIUM BOVIS* BACILLUS CALMETTE-GUERIN




(57) Abstract: The invention relates to a liposome comprising a chloroform soluble and extractable total polar lipid of *Mycobacterium spp*, particularly a chloroform soluble extractable total polar lipid of *Mycobacterium spp* BCG. The chloroform soluble and extractable polar lipid may comprise at least one of phosphatidylinositol (PI), phosphatidylinositol mannoside ($PIM_1$), phosphatidylinositol dimannoside ($PIM_2$), mono and dipalmitoylated forms of $PIM_1$ and $PIM_2$, phospholipid of 899 m/z, phosphatidylethanolamine and cardiolipid. The liposome may be prepared by drying chloroform soluble and extractable lipid and then hydrating said dried lipid at a temperature of 65 to 75 °C in water or phosphate buffered saline (PBS). The liposome may be used, for example, to activate dendritic cells to secrete cytokines and modulate an immune response in a mammal, or to direct an immune response to confer protection against a pathogen or a cancer.



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Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**

*without international search report and to be republished upon receipt of that report*

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

# VACCINE ADJUVANT PROPERTIES OF LIPOSOMES FORMED AT ELEVATED TEMPERATURES FROM THE POLAR CHLOROFORM EXTRACTABLE LIPIDS FROM *Mycobacterium bovis* Bacillus Calmette-Guérin

## FIELD OF THE INVENTION

This invention relates to the use of the chloroform-extractable polar lipids from the human vaccine strain of *Mycobacterium bovis* BCG, and other *Mycobacteria* with similar lipids, to prepare liposomes with immunomodulatory and adjuvant activity to promote an immune response to an associated antigen. Total polar lipids of BCG or purified lipid fractions PI, (phosphatidylinositol), $PIM_1$ (phosphatidylinositol mannoside), $PIM_2$ (phosphatidylinositol dimannoside) and their palmitoylated forms, or acylated-phospholipids of 899, 1139 and 1155 m/z are used to form liposomes at elevated temperatures and to activate antigen presenting cells in specific ways. The invention more specifically relates to vaccine development by providing a stable vehicle for antigen delivery to antigen presenting cells using immunostimulatory, chloroform extractable, polar BCG glycerolipids, resulting in enhancement of MHC class I and class II responses in an animal.

## BACKGROUND OF THE INVENTION

Historically, human vaccines have consisted of live attenuated viral or bacterial pathogens. Patient acceptance and safety is a concern based on possible side-reactions of complex and ill-defined vaccines, and the possibility for reversion to virulence. A more current approach is to use defined, highly purified antigens. Side-reactions are minimized, but the efficacy of these subunit vaccines is generally poor because of a loss in immunogenicity when the antigen is purified. A further difficulty is efficiently targeting both the antigen and adjuvant to precisely the same antigen presenting cells. Further, the lack of efficacy may be explained by an inappropriate immune response, because protection may require that either humoral, cell-mediated or cytotoxic T cell (CTL) responses predominate depending on the pathogen in question. For example, protective immunity against anthrax is thought to require only a humoral response, whereas, a protective vaccine against

intracellular pathogens such as *M. tuberculosis* or cancers require a strong CTL response. The use of Alum as an adjuvant (approved for human use) is based on forming a complex with antigen to give a depot effect, resulting in only a Th2 response, and not CTL. Further, local reactions may occur at the injection site with aluminum-based adjuvants such as Alum (Koike *et al.* 1998).

Other adjuvant systems such as archaeosomes (Krishnan *et al.* 2001) and immunostimulating complexes (ISCOMS) are especially suited as cell-mediated adjuvants, but give only moderate antibody responses. ISCOMS have issues related to toxicity of saponin preparations used in their construction, and in use with water-soluble antigens (Bowersock and Martin 1999). In cases where the antigen and adjuvant are not co-delivered as a particulate system, inefficiency occurs in antigen delivery to the same antigen presenting cells activated by the adjuvant. Many delivery systems also require co-adjuvants such as Quil A or Lipid A (ex. ISCOMS, conventional liposomes) with expense, stability, and toxicity issues associated with their use. Ease of production and costs can be an issue for many of these adjuvant systems. Finally, lack of retention of the encapsulated antigen will make a vaccine vesicle system ineffective.

*Mycobacteria* spp. are often associated with pathogenesis and are best known as causative agents for tuberculosis (*M. tuberculosis*), leprosy (*M. leprae*), and as opportunistic pathogens (*M. avium*). The ability of the immune system to respond to mycobacterial cells, or their components, has been an area of keen interest for decades because of the pathogenicity associated with this genus.

The current vaccine against tuberculosis in humans is the culture of *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) that became attenuated during passage in laboratory medium. Although the exact reasons for attenuation are still being researched (Behr *et al.* 2000), it was discovered early that heat-killed whole cells of *Mycobacterium tuberculosis* mixed with oil and an antigen resulted in strong adjuvant activity. This became known as Freund's Complete adjuvant (FCA) and has been used in many laboratories to promote a strong antibody, as well as a strong cytotoxic T cell (CTL) response (Skinner *et al.* 2001) to a protein antigen. Active components in FCA include muramyl dipeptide and trehalose 6,6'-dimycolate from the cell wall (Retzinger *et al.*1981).

Freund's Complete adjuvant is toxic causing acute inflammation, granulomas, and chronic toxicity (Retzinger *et al.*1981) and is unacceptable, therefore, for human or veterinary use.

The *Mycobacterium* spp. surface is composed of the cytoplasmic membrane surrounded by a cell wall made of mycoloyl arabinogalactan covalently attached to peptidoglycan, and associated lipoarabinomannan (LAM) (Chatterjee and Khoo 1998). All strains have these layers although the outer layer appears to differ in structural detail among strains (Ortalo-Magné *et al.*1996). Lipids comprise part of these various outer layers and account for up to 60% by weight of the mycobacterial cell wall. This includes the mycolyl-arabinogalactan-peptidoglycan, covalently linked polymer, and several types of "extractable"lipids. "Extractable" lipids found in various strains include: (1) trehalose-containing glycolipids, (2) glycopeptidolipids, (3) phenolic glycolipids, (4) lipooligosaccharides, (5) phosphatidylinositol mannosides (PIMs), (6) phosphatidylethanolamine, and (7) triacylglycerols (Wang *et al.* 2000; Besra and Brennan 1994). The completed structures for novel palmitoyl and dipalmitoyl-PIMs has been reported only recently (Gilleron *et al.* 2001). Further, these authors showed that phosphatidylinositol (PI) had the same ability as $PIM_1$ and $PIM_2$ (all apparently adsorbed on Alum) to induce recruitment of Natural Killer T cells, indicating no difference in biological response with addition of mannose residues to PI (Gilleron *et al.* 2001). This biological effect is in direct contrast to the stimulation of dendritic cells to secrete IL-12 by PIMs, and not PI, as shown in the current invention.

LAMs represent the mycobacterial counterpart to Gram-negative lipopolysaccharides. These lipids are composed of a phosphatidylinositol anchor, a mannan core, an arabinan domain, and also mannooligosaccharide caps in the case of ManLAMs (Chatterjee and Khoo 1998). LAMs exert their effects on the immune system in several ways. For example, LAM isolated from actively growing mycobacteria activated cells expressing a Toll-like receptor 2 (TLR2) in a TLR-dependent fashion, but LAM isolated from BCG could not (Means *et al.* 1999). LAM is a water-soluble polymer and would not, therefore, be a component of the chloroform-soluble lipids used herein (Nigou *et al.* 1997).

Several other lipids of mycobacteria have immunomodulatory activity. The phenolic glycolipid trehalose 6,6'-dimycolate (cord factor) is an active component in FCA capable of promoting an antigen-specific CTL response (Skinner *et al.* 2001), and

moderate antibody titres when injected with an antigen in oil (Koike *et al.* 1998). This contrasts to the current invention in 1) liposomes were not used 2) antibody titres were not high with cord factor and 3) cord factor was absent from the lipids used in this invention.

Both the phenolic glycolipids and glycopeptidolipids have been shown to be on the cell surface and to be capable of down-regulating the immune system of the host during infection (Ortalo-Magné *et al.* 1996). This down-regulation by surface lipid accounts, in part, for the success mycobacterial pathogens enjoy in evading the normal host response to invasion. This again is contrary to, teaching away from the current invention.

## SUMMARY OF THE INVENTION

Liposomes form at elevated temperatures above 55°C, and preferably 65°C to 75°C, from the chloroform-soluble total polar lipid fraction of *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), and its purified lipid components. Total polar lipids were separated by thin layer chromatography into eight fractions and characterized by specific spray reagents and mass spectrometry. Dendritic cells exposed to BCG total polar lipid liposomes were activated to excrete inflammatory cytokines, whereas lipids from commercial sources were relatively inactive. Dendritic cell activating activity for IL-12 secretion was localized to the phosphatidylinositol mannosides with a mannose residue ($PIM_1$ and $PIM_2$) and their acylated forms, and to novel BCG acylated-phospholipids of m/z 899, 1139 and 1155. Indeed, activity was considerably higher in these purified BCG lipid liposomes than in the BCG total polar lipid liposomes. In contrast, BCG phosphatidylinositol activated dendritic cells to secret tumor necrosis factor (TNF) (in absence of mannose residues) at amounts higher than BCG total polar lipid liposomes. This stimulation depended on the presence of the fatty acyl chain, tuberculosteric acid, characteristic of mycobacterial lipids, as PI from soybean was without effect. Cardiolipid, and phosphatidylethanolamine formed a major portion of the BCG total polar lipids yet in purified form had low activating activities. Mice immunized with a protein antigen entrapped in BCG total polar lipid liposomes produced both MHC class I and class II responses. Similar trials with liposomes composed of the total polar lipids extracted from another Gram-positive bacterium, *Bacillus firmus*, or PC/PG/cholesterol revealed that BCG liposomes had much superior adjuvant properties. A vaccine prepared from BCG liposomes gave protection to mice upon challenge with tumor cells.

According to one aspect of the invention a method is provided for forming liposomes from the total polar, chloroform extractable lipids of BCG or from any of the lipid components therefrom.

It is another object of this invention to utilize liposomes prepared from one or more of the chloroform extractable lipids from BCG as novel, immunomodulating carriers for antigens in vaccines, to induce immune responses in a vaccinated animal and protect against infection

Yet another object of the invention is to use BCG liposome vaccines to protect the vaccinated animal against cancer.

According to a further aspect of the invention, BCG lipids $PIM_1$, $PIM_2$ or their acylated forms are used in liposome vaccines to activate the antigen presenting dendritic cells and induce secretion of IL-12 (Interleukin-12).

An object of this invention is to use BCG lipid PI in liposome vaccines to activate the antigen presenting cells to secrete TNF.

Yet another object of the invention is to use the novel saturated glycerolipids of BCG with at least one tuberculosteric fatty acyl chain per molecule to form liposomes with prolonged shelf life and stable to the conditions found upon vaccination of an animal.

According to still another aspect of the invention BCG lipids are used to induce the excretion of inflammatory cytokines to promote an immune response.

According to another aspect of the invention, a method is provided to elicit an antigen specific MHC class I-restricted cytotoxic T cell response and an antigen specific MHC class II-restricted response in an animal, comprising administering to the animal a vaccine liposome composition prepared from BCG polar, chloroform extractable lipid and an antigen

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a fast atom bombardment mass spectrometer (FAB MS) spectrum of the chloroform extractable, total polar lipids of BCG. Signals are identified as PI (phosphatidylinositol); PE (phosphatidylethanolamine); $PIM_1$ (phosphatidylinositol monomannoside); $PIM_2$ (phosphatidylinositol dimannoside); and fragment ion [PA]

(phosphatidic acid). Assignments are shown in the figure for the total number of carbon atoms in the sn-1,2 glycerolipid fatty acyl chains: followed by number of unsaturations.

Figure 2 shows the separation of BCG total polar lipid into 8 lipid fractions by thin layer chromatography. Fractions are numbered 1 to 8, from most polar to least polar. Lipids (applied at the bottom of the plate) are BCG (chloroform extractable, total polar lipids), and PI, PS (phosphatidylserine), PG (phosphatidylglycerol), and PE (phosphatidylethanolamine) are reference standards. An acidic solvent was used to develop the plate. Lipids were located by spraying with the Zinzade phosphate spray reagent, so the spots shown are all phospholipids.

Figure 3 is a FAB MS spectrum of lipid fraction 1 showing that it contains saturated $PIM_2$ and a lipid of m/z 899. As shown, both lipids yield typical PA fragmentation to generate m/z 688+H, wherein the sum of carbon atoms in both sn-1,2 chains and their state of saturation is C35:0. The lipid structure of 899 m/z is shown to be a phosphatidylglycerol phosphate (PGP) acylated with a moiety of m/z 57.

Figure 4 is a FAB MS spectrum of lipid fraction 2 showing that it contains saturated palmitoyl-$PIM_2$ (Palm- $PIM_2$). Smaller amounts corresponding to about 12% $PIM_1$ and palmitoyl- $PIM_1$ are present also.

Figure 5 is a FAB MS spectrum of lipid fraction 3 showing that it contains saturated pure PI. About 80% of PI has C19:0 and C16:0 chains and much of the remaining PI has C19:0 and C15:0 chains.

Figure 6 is a FAB MS spectrum of lipid fraction 4 showing that it contains saturated dipalmitoyl-$PIM_2$ with sn-1,2 C19:0 plus C19:0 or C19:0 plus C16:0 glycerolipid chains. Some PI is found in this fraction also.

Figure 7 is a FAB MS spectrum of lipid fraction 5 showing that it contains saturated palmitoyl- $PIM_1$, PI, and about 12% palmitoyl-$PIM_2$.

Figure 8 is a FAB MS spectrum of lipid fraction 6 showing that it contains saturated novel lipids of m/z 899.1, shown as acyl-PGPs (acyl-phosphatidylglycerol phosphate). In these PGP lipids the terminal phosphate is protonated, as indicated in the structure by a negative charge on only the phosphate closest to the glycerol backbone. A longer acyl-chain form of 1139.2 m/z corresponds to loss of the methyl group (on the phosphate) in the structure shown and replacement by a palmitoyl chain. Another related structure is 16 larger than 1139 indicating hydroxylation of one of the three acyl-chains.

Figure 9 is a FAB MS spectrum of lipid fraction 7 showing that it consists of saturated pure PE lipids of various chain lengths.

Figure 10 is a FAB MS spectrum of fraction 8 showing that it consists of unsaturated cardiolipid.

Figure 11 is a FAB MS spectrum of the total polar lipids extracted from *Bacillus firmus* and defines these lipids as primarily phosphatidylglycerols and cardiolipids.

Figure 12 shows that mice immunized subcutaneously at 0 and 3 weeks with antigen (OVA) entrapped in BCG liposomes provides protection against challenge with EG.7 (OVA expressing) tumor cells. It further shows that antigen-free BCG liposomes exert some innate protective effect against tumor growth seen as a delay in the onset of tumors.

## DETAILED DESCRIPTION OF THE INVENTION

Liposomes are closed spherical vesicles composed of a lipid bilayer with polar headgroups exposed to inner and outer surfaces and the lipid chains forming the interior part of the bilayer. Water-soluble drugs or antigens are either bound to the surface or entrapped in the fluid space within the liposome, whereas hydrophobic molecules tend to associate with the lipid layer.

In the present invention the total lipids from fresh BCG cells are extracted with ambient temperature methanol/chloroform/water (2:1:0.8, v/v), and the polar chloroform-extractable lipids separated from neutral lipids as the cold-acetone insoluble fraction. We show for the first time that this total polar lipid fraction, and purified lipids therefrom such as PIMs and palmitoyl-PIMs, will form liposomes provided the temperature is sufficiently high and preferably 65-75 °C. Animals may then immunized with BCG liposomes associated with one or more protective antigens to confer protection to pathogens or cancer where a strong immune response is required, or for the production of high antibody titres for research purposes. Adjuvant activity includes not only the MHC class II mediated Th2 and antibody arm of the immune response, but also MHC class I responses evident by induction of CTL responses and INF-gamma secreting CD8+ T cells in the Elispot assay and by showing that a BCG liposome vaccine can protect in a mouse tumor model. There have been no previous reports to our knowledge demonstrating either

liposome construction from the lipids of mycobacteria, and specifically from the polar-extractable lipids, or reports of immunomodulation by such liposomes, thus obviating the need to include additional adjuvants in a BCG liposome vaccine.

Because temperatures above 55°C are required to prepare liposomes efficiently from BCG total polar lipids, or its purified components, it follows that at lower temperatures such as body temperatures the liposome membranes would pass from liquid crystalline phase to solid phase. In solid phase the membranes would be less leaky to entrapped antigen and stability of the liposomes would be enhanced resulting in an improved vaccine carrier. Further, several of the purified BCG lipids when converted to liposomes have immunostimulatory activity, shown herein by the activation of dendritic cells, the most potent cell type for processing and presenting antigen to T cells. The active lipids are composed of a glycerol backbone linked sn-1,2 with saturated fatty acyl C19:0 chains of tuberculosteric (unique to mycobacteria) and C16:0 palmitic acids, and a phosphoinositol headgroup at the sn-3 position linked to 1 or 2 mannose sugar residues (PIM1, PIM2), sometimes mono- or dipalmitoylated.

These active lipids may be obtained from BCG cells that are grown easily with high yield, or alternatively it may be appreciated they may be chemically synthesised, as their structures are known. Further, the tuberculosteric acid found in *Mycobacteria* species is a methyl-branched, long-chain, fully saturated fatty acyl chain, and as such is predicted to contribute to liposome stability. Indeed, the only BCG lipid that has unsaturation is the cardiolipids fraction 8.

## MATERIALS AND METHODS

*Source of bacteria and growth*

*Mycobacterium bovis* (BCG) Pasteur strain was obtained from Dr. Robert North (Trudeau Institute, USA) and grown aerobically in 1-liter shake flasks containing standard complex medium. *Bacillus firmus* was purchased from the American Type Culture Collection (ATTC 14575) and grown aerobically on Nutrient Broth 8 g/l, Yeast Extract 3 g/l (Difco Laboratories, Mich.), urea 1.5 g/l and Bactopeptone 1 g/l at 30 °C.

*Source of cell cultures*

EL-4 and EG.7, a subclone of EL-4 stably transfected with the OVA gene, were obtained from the ATTC, and maintained and grown as described before (Krishnan *et al.* 2000).

*Lipid extraction*

Briefly, total lipid extracts were obtained from frozen-thawed cell pastes of *B. firmus*, or from fresh cell paste of *M. bovis* by adding a one-phase solution of methanol, chloroform, and water (2:1:0.8, v/v) in a ratio of 15 g cell dry weight/l. After 16 h the cellular debris was collected by centrifugation and re-extracted twice more. Extracts were pooled and made biphasic by addition of chloroform and water by the Bligh and Dyer method previously described (Sprott *et al.* 1995). Polar lipids in the chloroform bottom phase were freed of neutral lipids by differential solubility in cold acetone (Sprott et al. 1995). Polar lipids, insoluble in cold acetone, were dried and dissolved into chloroform as the chloroform-extractable total polar lipids. BCG total polar lipids dissolved in chloroform were filtered using a 0.45 μm nylon syringe-filter, to ensure there was no carry over of whole cells into the lipid extract.

*Liposome preparation*

1) *B. subtilis* liposomes - about 30 mg of total polar lipids in chloroform were dried under a nitrogen stream, and hydrated by adding 3.0-ml of pyrogen-free water. Hydration was allowed to proceed for 2-3 h at 35 °C with shaking prior to the addition of 10 mg ovalbumin (OVA)/30 mg lipid. Average vesicle diameters were decreased from 80 to100 nm in a sonic bath. Preparations were then freeze-dried and re-hydrated in phosphate buffered saline (PBS, 10 mM potassium phosphate plus 160 mM NaCl, pH 7.1). OVA was removed by centrifugation and three washes with PBS. The final liposome pellets were re-suspended into PBS, and liposomes filter-sterilized using syringe-driven 0.45 μm filters (Millipore, MA). Entrapped OVA was quantified after lipid removal by the SDS-Lowry colour development method as described before (Krishnan *et al.* 2000) and dry weights determined. Pyrogen-free sterile water was used throughout.

2) Commercial lipids – L-α-dimyristoylphosphatidylcholine (DMPC), L-α-dimyristoylphosphatidylglycerol (DMPG), L-α-phosphatidylinositol (soybean, PI),

cardiolipid (bovine heart), and L-α-dipalmitoylphosphatidylethanolamine (DPPE) were purchased from Sigma. Liposomes were prepared as described for total polar lipids of *B. subtilis*, except for the omission of OVA.

3) BCG liposomes – about 30 mg of total polar lipids in chloroform were dried under a nitrogen stream followed by 1-h under vacuum. Hydration was routinely done by adding 3-ml of pyrogen-free water containing the antigen (for example 10 mg OVA) and incubating for 2-3 h at 65°C with shaking. To investigate the effect of temperature on liposome formation, hydration was allowed to occur at 35°C to 75°C, in 10°C steps. Average vesicle diameters were decreased between 80 - 100 nm in a sonic bath at 65°C. Preparations were then freeze-dried and re-hydrated in PBS at 65°C. Liposomes were left overnight at 4°C to anneal, then any OVA not associated with the liposomes was removed by ultracentrifugation and washing liposomes with PBS thrice. The final liposome pellets were re-suspended into PBS, and liposomes filter-sterilized using 0.45 μm filters. Entrapped OVA was quantified after lipid removal and dry weights determined, as above. Average diameters were measured in a 5 mW He/Ne laser particle sizer (Nicomp Model 370). BCG liposomes were made from isolated lipid fractions 1 to 8 by the above method, except for BCG PE fraction 7. PE liposomes were made by including 80 mole % DMPC, as PE lipids in general do not make liposomes in pure form. This also was the case for BCG PE.

*Lipid analysis*

Polar lipid extracts were analysed by fast atom bombardment mass spectrometry (FAB MS) with a JEOLJMS-AX 505H instrument operated at 3 kV in negative ion mode. The xenon gun was operated at 10 kV. Current-controlled scans were acquired at a rate of 10-s full scale. A mixture of triethanolamine and Kryptofix® (Sigma) was used as the matrix. Staining for functional groups was done after separating the polar lipids on pre-coated 0.25 mm silica gel 60 thin-layer plates (Merck) developed with an acidic solvent chloroform/methanol/acetic acid/water (85:22.5:10:4, v/v) or basic solvent chloroform/methanol/7 N ammonium hydroxide (60:35:8, v/v). Lipid spots were characterized using the phospholipid (Zinzade's reagent), glycolipid (α-naphthol), aminolipid (ninhydrin), and total lipid (sulfuric acid char reagent) sprays described in Kates (1986). For sugar analysis lipids were first hydrolysed with 2 M trifluoroacetic acid

for 2 h at 100°C. D-ribose was then added as an internal standard, and alditol acetate derivatives prepared for identification and quantification by gas chromatography-mass spectrometry (GC MS) (17). The total carbohydrate content of each lipid extract was determined by Anthrone reaction using D-glucose as the standard.

*Dendritic Cell (DC) isolation and activation*

Bone marrow derived dendritic cells were prepared as described before (Krishnan *et al.* 2001), and were consistently > 80% $CD11c^+$ by flow cytometry. Briefly, bone marrow was flushed from the femurs and tibias of C57BL/6 mice, and single cell suspensions made. Cells obtained were cultured ($1X10^6$/ml) in RMPI medium supplemented with 8 % fetal bovine serum, FBS (R8) and 5 ng/ml of recombinant murine GM-CSF (ID Labs, London, ON, Canada) for 6-8 days at 37°C in 8% $CO_2$. Non-adherent cells were removed at days 2 and 4 of culture, and fresh R8 plus GM-CSF was added. Dendritic cells were harvested on days 6-8 as non-adherent cells. Dendritic cells ($10^5$) were incubated *in vitro* with various concentrations of antigen-free archaeosomes or lipopolysaccharide (LPS, *E. coli*, Sigma), in triplicate in 96-well tissue culture plates, for 72 h at $37^0$C, 8 % $C0_2$, in a humidified atmosphere. At 72 h, activation of the cells was assessed by measurement of MTT (dimethylthioazol diphenyltetrazolium bromide) uptake and the supernatants were collected and IL-12 was assayed by sandwich ELISA (Mosmann & Fong 1989). TNF was assayed by a bioassay referenced in Krishnan *et al.* 2001.

*Immunizations*

Female, C57BL/6 mice, 6-8 weeks of age, were immunized subcutaneously at the base of the tail at 0 and 21 days. Immunizations were with 15 μg OVA either with no adjuvant, or OVA entrapped in liposomes prepared from the total polar lipids extracted from BCG or *B. firmus*. In some cases FCA was included in the first immunization and Freund's incomplete adjuvant (FIA) in the second at 62% strength with 15 μg OVA in PBS.

*Analysis of humoral response*

Sera were collected from blood obtained from the tail veins of mice and analysed for anti OVA antibodies. The antibody titres were determined by indirect antigen-specific ELISA. Briefly, ELISA plates (EIA microtitration plates, 96-well flat bottom, ICN Biomedicals Inc., Aurora, OH) were coated with antigen in PBS (10 μg/ml), and serial two-fold dilutions of serum (from individual mice) were assayed in duplicate. HRP-conjugated goat anti-mouse immunoglobulin (IgG + IgM) revealing antibody (Caltag, San Francisco, CA) was used to determine total antibody titres of sera. The reactions were developed with ABTS microwell peroxidase system (Kirkegaard and Perry Laboratories, Gaithesburg, Maryland) and absorbance determined at 415 nm after 15 min. Antibody titres are represented as endpoint dilutions exhibiting an optical density of 0.3 units above background.

*CTL assays*

For CTL assays, 30 X $10^6$ spleen cells were cultured with 5 X $10^5$ irradiated (10,000 rads) EG.7 cells in 10 ml of RPMI plus 8 % FBS containing 0.1 ng/ml IL-2, in 25 $cm^2$ tissue culture flasks (Falcon), kept upright. After 5 days ($37^0$C, 8% $CO_2$), the cells recovered from the flask were used as effectors in a standard $^{51}$Cr-release CTL assay and % specific lysis against EG.7 targets determined (Krishnan *et al.* 2001).

*ELISPOT assay*

Enumeration of IFN-γ secreting cells was done by ELISPOT assay (Vijh and Pamer, 1997). Briefly, spleen cells were incubated in anti-IFN-γ antibody coated ELISPOT plates in various numbers (in a final cell density of 5x$10^5$/well using feeder cells) in the presence of IL-2 (1 ng/ml) and RPMI media or $OVA_{257-264}$ (10 μg/ml) for 48 h at $37^0$C, 8 % $CO_2$. The plates were subsequently blocked, incubated with the biotinylated secondary antibody ($4^0$C, overnight), followed by avidin-peroxidase conjugate (room temperature for 2 h). Spots were revealed using di-amino benzidine.

*Tumor model*

A murine solid tumor model was used to assess the relative protective potential of $CD8^+$ T cells induced by BCG TPL liposomes with OVA entrapped. Mice were injected

twice at 0 and 21 days with OVA, 15 μg/0.1 ml injected per mouse, given subcutaneously. EG.7 cells ($5 \times 10^6$) expressing OVA (in PBS plus 0.5 % normal mouse serum) were injected in 0.1 ml in the shaved lower dorsal region, 9 weeks post first injection. From day 5 onwards, detectable solid tumors were measured using callipers. Tumor size, expressed in $mm^2$, was obtained by multiplication of diametrically perpendicular measurements.

## RESULTS AND DISCUSSION

### Lipid extraction from BCG

In this invention centrifuged cell pellets of BCG (10 g) are extracted at ambient temperature by stirring for 24 h with 1 liter of 1-phase Bligh and Dyer consisting of methanol/chloroform/water (2:1:0.8, v/v). The mixture is centrifuged at 10,500 x g for 15 min and supernatant and pellet fractions separated. The pellet fraction is extracted twice more as above and the three supernatants combined. Upon storing at 4°C a cloudy white precipitate forms and is removed by centrifuging at 4,100 x g for 15 min. This supernatant contains most of the chloroform extractable lipids. The small pellet removed is extracted again with 1-phase and the supernatant from this extraction combined with the chloroform extractable lipids. A volume of chloroform and water each equal to the total volume of the chloroform extractable lipids divided by 3.8 is added to obtain a 2-phase system in glass separatory funnels. The bottom chloroform phase containing the desired lipids is removed, and two 200-ml volumes of chloroform are used to wash the upper methanol-water phase. These chloroform washes are combined with the first chloroform phase. Also combined with the chloroform phases is chloroform recovered by centrifuging the milky emulsions formed at the interface of the two phases. Total polar lipids are recovered from the chloroform phases concentrated by flash evaporation by precipitating upon adding 20-volumes of ice-cold acetone. The pellet obtained is washed twice with ice cold acetone, dissolved in chloroform, and finally filtered through nylon 0.45 μm filters to obtain chloroform extractable total polar lipids (TPL). The total lipids account for about 10.2% of the starting BCG cell weight, of which 65% is total polar lipids and 35% acetone soluble lipids. A typical FAB MS spectrum of the total polar lipids is shown in figure 1. Dominant lipids were assigned as PE, PI, $PIM_1$, palmitoyl-$PIM_1$, $PIM_2$, palmitoyl- $PIM_2$, and cardiolipid. Dominant fatty acid carboxylate anions generated from the polar lipids during MS analysis are C16:0, C19:0 and C18:1. The signal of m/z 297.3 corresponds to the *M. tuberculosis* C19:0 fatty acid, 10-methyloctadecanoate, known as tuberculosteric acid (Leopold and Fisher 1993). A headgroup analysis shows mannose to be the major sugar present in about equal amount to inositol (Table 1).

A hot ethanol soluble lipid fraction may be obtained from the cell pellet above, already extracted three times with 1-phase Bligh and Dyer solution, by dispersing into 50% ethanol and refluxing at 65°C for 8 h. The mixture is centrifuged at 10,500 x g for 20

min and the supernatant flash evaporated to remove the ethanol. The remaining liquid is extracted with 1-phase Bligh and Dyer solution and made 2-phase to recover the hot ethanol lipids in the chloroform phase. Hot ethanol extracted lipids were similar to TPL in mannose and inositol content and represent roughly half of the mannose and inositol recovered in TPL lipids (Table 1). Thin layer chromatography and FAB MS show the same lipids present as in TPL. Thus, although only the chloroform extractable TPL is used herein, it is appreciated that TPL yield may be increased by combining, or replacing with a hot ethanol extraction. It may also be appreciated that a hot ethanol extraction may be used as an alternative to the Bligh and Dyer method to obtain essentially the same lipids using less costly and less toxic solvent.

**Purification and characterization of the BCG TPL lipids**

Thin layer chromatography is used to separate TPL into 8 fractions, all of which stain positively for phosphate (Fig. 1). Fraction 4 has similar mobility to standard PI and fraction 7 corresponds to a PE standard. Staining reactions further define these 8 lipid fractions (Table 2). Lipids 1, 2, 4 and 5 are phosphoglycolipids, 3, 6 and 7 are phospholipids, and lipid 7 is a phosphoaminolipid.

To purify components of TPL, BCG TPL is applied as a band (6 mg/plate) and separated in this way into the 8 fractions by locating bands with iodine vapor and recovering the chloroform soluble lipids from the removed adsorbent. Bands 3 and 4 merge and may be recovered together, then separated and recovered using another thin layer plate and an alkaline solvent. The relative abundance of each recovered fraction 1 to 8 is shown in Table 2.

The 8 lipid fractions are defined structurally by FAB MS analysis in figures 3 to 10. Fraction 1 consists of $PIM_2$ and an 899.5 m/z lipid. Both have C19:0 and C16:0 chains as only these two chains are seen as carboxylate anions (Figure 3). The 899.5 m/z lipid is clearly in low relative amount, as it is not seen in a spectrum of TPL (Figure 1). An acyl-PGP lipid structure consistent with the above spectrum is shown in figure 3, in which the acyl group must be 57 m/z (either a propionic fatty acyl or butyl group). In these structures of BCG lipids, glycerol moieties are shown in 'stick' form and tuberculosteric acid chain position is sn-1 based on Gilleron *et al.* (2001).

Lipid fraction 2 is primarily palmitoyl- $PIM_2$ with C19:0 and C16:0 chains (Figure 4), and fraction 3 is pure PI (Figure 5). In the case of PI most molecules have C19:0 plus C16:0 chains, with the bulk of the remaining PI having C19:0 and C 15:0 chains. Fraction 4 is a phosphoglycolipid defined by FAB MS as dipalmitoyl- $PIM_2$ of various sn-1,2 chain forms ranging from C35:0 to C38:0 (Figure 6). PI is detected also in this fraction. Palmitoyl- $PIM_1$ and PI in about equal amount comprise fraction 5 (Figure 7) a phosphoglycolipid fraction of only 3% abundance in BCG TPL (Table 2). Fraction 6 is a phospholipid of m/z 899.1 (Figure 8) comprising only 1.2% of TPL. The most dominant PA fragment ion is 731.2 m/z indicating a methyl-PGP with sn-1,2 tuberculosteric acid fatty acid chains. However, other PA fragment anions, and fatty acid carboxylate signals, indicate PGP molecules with other sn-1,2-chains and acyl moieties on the terminal phosphate to total a m/z of 899.1, the primary signal for the molecular anion. Clearly, fraction 7 is pure PE with several sn-1,2 chain combinations, primarily C18:0 plus C16:0 (C34:0). PA fragment ions at m/z 647.1 and 675.1 confirm the PE assignments and correspond to fragments from C32:0 and C34:0, respectively. Finally, mobility of thin layer plates, staining reaction, and FAB MS identifies fraction 8 as a cardiolipid with mainly C18:1 and C16:0 chains and a molecular anion signal of 1403.2 m/z (Figure 10).

**Liposome formation from BCG chloroform extractable polar lipids**

Contrary to expectation BCG TPL (total polar lipid) did not form liposomes at the normal growth temperature of *M. bovis* BCG, namely 37°C. TLP lipids were dried from solvent and liposomes monitored by phase microscopy after addition of water, or PBS buffer, at 35, 45, 55, 65, and 75°C. Liposomes did not form well at 55°C or less, resulting in clumps of lipid, but elevating the temperature to 65 or 75°C resulted in a dramatic formation especially when water was used for hydration. In this invention then, chloroform extractable BCG TPL is hydrated preferably at 65°C in the presence of antigen to form multilamellar liposomes. Smaller liposomes are produced, if desired, by size reduction at preferably 65°C using a bath sonicator. Other methods of size reduction could be used if the temperature is 65°C. Entrapment of antigen may be improved by lyophilization and rehydration of the liposome powder in water at 65°C, followed by PBS. Liposomes are then annealed and any unentrapped antigen removed as described in

Materials and Methods. Average diameters were 230 ± 136 nm with OVA loadings in 3 preparations ranging from of 33 to 67 μg/mg dry weight of liposomes. Average diameters of BCG liposomes made from the purified lipid fractions are shown in Table 3. Those skilled in the art will appreciate that the various methods described in liposome formation should apply to these BCG lipids providing care is taken to achieve the required temperature, preferably 65°C.

**Activation of dendritic cells by BCG TPL liposomes and liposomes prepared from purified lipids fraction 1 to 8**

Because dendritic cells represent the major antigen presenting cells in mammals, they are the preferred cells for *in vitro* adjuvant testing. Bone marrow dendritic cells were cultured with zero (R8 medium only) to 10 μg dry weight liposomes/ml of R8 medium. Liposomes tested are shown in table 3 and include several made from commercial lipids. After 72 h the numbers of viable cells were quantified by the MTT assay and secreted inflammatory cytokines assayed in the culture supernatants. Liposomes at 10 μg/ml giving an MTT of >25% above the control R8 medium, were limited to BCG TPL liposomes, and purified BCG lipid fractions 3 (PI) and 6. All liposomes made from non-BCG lipids; namely, DMPC, DMPG, PI from soybean, DPPE + DMPC, and cardiolipid from brain, were without significant activity, measured as IL-12 secretion from dendritic cells. However, PIM fractions 1 and 2, as well as fraction 6, induced secretion of IL-12 several-fold above that induced even by BCG TPL liposomes (at 10 μg/ml). Further, induction of IL-12 secretion required the addition of at least the complexity of 1 mannose unit to BCG PI, as purified BCG PI (fraction 3) was relatively inactive in this regard.

Further unexpected results are seen in the case of induction of TNF (tumor necrosis factor) secretion from dendritic cells. BCG TPL liposomes were again active. However, contrary to the effects on IL-12 secretion, TNF secretion occurred with purified BCG PI liposomes, and not with other BCG lipid liposomes or commercial lipid liposomes, clearly showing that BCG PI is the active lipid in BCG TPL accounting for TNF secretory activity. The fact that PI from soybean (with no tuberculosteric acid chains) was inactive, while BCG PI was highly active points solidly to the tuberculosteric acid (C19:0) as a key structural difference to explain active versus inactive PIs. Clearly, the lipids in BCG TPL have very different biological effects on activation and cytokine secretion from dendritic

cells and consequently on the type of immune response obtained. Also clear, preparing liposomes using various combinations of BCG polar lipids is indicated as a mechanism to direct the type of immune response obtained to an entrapped antigen.

Phosphatidylethanolamines (PEs) are known generally as fusogenic lipids, capable of promoting fusion of membranes, and account for about 25% of the lipids in BCG TPL (Table 2). To mount a CTL immune reaction it is first necessary to deliver antigen to the cytosol of antigen presenting cells. It follows that inclusion of this lipid in a liposome with antigen entrapped, may aid in directing the immune response to MHC class I presentation of antigen and mounting a CTL response.

**Immune response in mice**

In a first example the adjuvant activity of BCG liposomes is compared to several other adjuvant systems. In one of these TPL liposomes from another Gram positive bacterium are included. The bacterium chosen was *Bacillus firmus* based on the observation that injections of these lipids into mice 5 days prior to infection with *Listeria monocytogenes* resulted in some short-term protection (Mára *et al.* 1992), presumably by activating the innate immune system. The TPL lipids of this bacterium extracted by the Bligh and Dyer method and collected as the acetone insoluble lipids, are characterized by FAB MS (Figure 11). In the case of *B. firmus* polar lipid extracts, m/z signals for the molecular anion of each lipid were assigned to a cluster of PG lipids with fully saturated sn-1,2 fatty acyl chains consisting of from 25 to 33 carbon atoms (sum of both chains) (Fig. 3). These assignments are consistent with the m/z of the carboxylate anions generated from the fatty acyl glycerochains during the analysis, which ranged from C13:0 to C17:0. Also, signals were found in the cardiolipid and LPG (lysophosphatidylglycerol) regions of the spectrum. An amino acid analysis confirmed the presence of small amounts of lysine in hydrolysates of *B. firmus* polar lipid extract, indicating the possibility of LPG and/or lysylcardiolipids (Fisher and Leopold 1999).

Table 4 represents a first example of an enhanced immune cytotoxic T cell (CTL) response raised in an animal to an antigen entrapped in BCG liposomes. BCG liposomes served to promote an immune response to the entrapped antigen that was similar to live BCG recombinant, and superior to Freund's adjuvant. Further, the TPL of another Gram

positive bacterium also with saturated glycerolipids formed liposomes with inferior properties to BCG liposomes, teaching away from the positive result with BCG liposomes.

Table 5 describes the humoral adjuvant activity of BCG liposomes to entrapped protein. First, injection of equivalent amounts of BCG liposomes and OVA produced no adjuvant activity, whereas entrapment resulted in humoral adjuvant activity comparable to the adjuvant Alum. The toxic adjuvant FCA was superior in potency and in maintaining the antibody titre for longer periods after vaccination. Live recombinant BCG expressing OVA *in vivo* produced CTL as expected (Dudani et al, 2002), but no antibody response. Finally, liposomes containing no BCG lipids gave very low antibody titres that improved as BCG lipids were incorporated at increasing amounts from 10 to 50%.

The effect of loading BCG liposomes with different amounts of antigen per mg liposomes is shown to be insignificant from the range of 15 μg antigen loaded in 0.22 to 1.8 mg (dry weight) of liposomes. This is shown in Table 6 for the MHC class I-restricted, $CD8^+$ T cell response. In A) results are shown of CTL assays and in B) numbers of IFN-gamma secreting precursor T cells in spleens of the variously immunized mice that specifically recognize the antigen in the original vaccination. Both assays show good adjuvant activity but no differences based on loading of the vaccine within this range.

**Antigen loaded BCG liposomes as protective vaccines**

Mice given large numbers of EG.7 tumor cells develop rapidly growing solid tumors reaching >250 $mm^2$ in all 5 mice in the naïve group within 12 days (Figure 12). Injections of antigen-free BCG liposomes resulted in a modest decline in tumor growth, where only 2 mice out of 5 developed tumors >250 $mm^2$ after 12 days. Furthermore, a clear delay in the onset of tumor growth is seen. Considerably more protection was seen for mice immunized with the BCG OVA liposome vaccine, where in all mice tumors were <250 $mm^2$ after 12 days and remained so for the duration of the study (for 4 mice out of 5).

Table 1. Percent relative abundance of inositol and mannose headgroups in BCG total polar lipids (TPL) and hot ethanol extracted lipids.

BCG cells were first extracted by the Bligh and Dyer method to generate TPL. These cells were extracted again with hot 50% ethanol to yield a hot ethanol lipids.

| | TPL | Hot ethanol |
|---|---|---|
| % carbohydrates | 5.62 | 2.79 |
| Mannose | 4.52 | 1.96 |
| Glucose | 1.05 | 0.73 |
| Galactose | 0.0 | 0.0 |
| Mannosamine | 0.0 | 0.0 |
| Glucosamine | 0.0 | 0.0 |
| Galactosamine | 0.0 | 0.0 |
| Arabinose | 0.062 | 0.099 |
| Inositol (% of Man) | 4.29 | 2.57 |

Table 2. Thin layer chromatography of the chloroform extractable total polar lipids from BCG cells into 8 fractions.

An acidic solvent was used to separate fractions 1, 2 and 5 to 8. In the case of fractions 3 and 4 the lipids were recovered together and run again on a second thin layer plate using a basic solvent to achieve separation. Staining reactions and recoveries are shown for each lipid fraction.

| Fraction | Phosphate stain | Sugar stain | Amino stain | % (w/w) abundance in TPL |
|---|---|---|---|---|
| 1 | + | + | - | 8.6 |
| 2 | + | + | - | 12.4 |
| 3 | + | - | - | 14.1 |
| 4 | + | + | - | 10.4 |
| 5 | + | + | - | 3.0 |
| 6 | + | - | - | 1.2 |
| 7 | + | - | + | 25.9 |
| 8 | + | - | - | 24.4 |

Table 3. Activation of bone marrow dendritic cells by antigen-free BCG liposomes.

The ability of liposomes prepared from BCG total polar lipid and BCG lipid fractions 1 to 8 are compared to liposomes made from commercial lipids. Background measurements for R8 medium alone were not subtracted from the values in the table, but were 0.542 (MTT), 9.3 (IL-12), and 0 (TNF). ND, not done.

| Liposome (diameter nm) | MTT (Absorbance) | | | IL-12 (ng/ml) | | | TNF (pg/ml) | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0.1 µg/ml | 1 µg/ml | 10 µg/ml | 0.1 µg/ml | 1 µg/ml | 10 µg/ml | 0.1 µg/ml | 1 µg/ml | 10 µg/ml |
| BCG TPL (414 ± 273) | 0.418 | 0.495 | 0.727 | 10.8 | 8.5 | 48.5 | 19 | 45 | 26 |
| BCG 1 (53 ± 34) | 0.374 | 0.321 | 0.574 | 4.2 | 3.0 | 168.4 | 0 | 1 | 0 |
| BCG 2 (48 ± 44) | 0.468 | 0.415 | 0.618 | 4.5 | 29.8 | 230.2 | 1 | 1 | 10 |
| BCG 3 (53 ± 35) | 0.448 | 0.382 | 0.774 | 2.5 | 2.8 | 36.6 | 46 | 80 | 146 |
| BCG 5 (162 ± 97) | 0.459 | 0.532 | 0.613 | 4.0 | 13.8 | 92.9 | 0 | 0 | 0 |
| BCG 6 (138 ± 111) | 0.438 | 0.501 | 0.745 | 7.3 | 26.6 | 147.2 | 0 | 5 | 25 |
| BCG 7+DMPC (68 ± 37) | 0.407 | 0.443 | 0.499 | 7.2 | 8.6 | 39.9 | 0 | 0 | 0 |
| BCG 8 (45 ± 24) | 0.439 | 0.604 | 0.896 | 3.2 | 5.2 | 41.6 | 0 | 0 | 32 |
| DMPC (270 ± 214) | 0.431 | 0.438 | 0.394 | 9.0 | 7.9 | 13.1 | 0 | 4 | 2 |
| DMPG (94 ± 49) | 0.421 | 0.386 | 0.404 | 3.6 | 4.8 | 8.4 | ND | ND | ND |
| PI soybean (29 ± 20) | 0.402 | 0.408 | 0.448 | 6.0 | 5.3 | 7.6 | 0 | 1 | 2 |
| DPPE + DMPC (135) | 0.372 | 0.388 | 0.439 | 7.6 | 5.4 | 3.4 | 4 | 12 | 20 |
| Cardiolipid (128 ± 73) | 0.419 | 0.429 | 0.609 | 6.8 | 6.6 | 10.2 | 11 | 14 | 5 |
| LPS | - | 0.564 | 0.562 | - | 187.9 | 182.7 | ND | 110 | 968 |

Table 4. Comparison of CTL activity in splenic cell cultures from mice immunized with OVA in various adjuvant systems.

Mice were immunized subcutaneously at 0 and 21 days with 15 μg OVA entrapped in BCG total polar lipid liposomes, mixed with Freund's adjuvant, or entrapped in liposomes prepared from the total polar lipids extracted from *B. firmus* (92 ± 48 nm diameter, loading 53 μg/mg). In the case of live BCG cells expressing OVA, only one subcutaneous injection was given containing $10^6$ cells. Spleens from duplicate mice were pooled for each analysis 10 weeks post first injection with exception of *B. firmus* taken 6 weeks post first injection. Lysis of control EL-4 cells not expressing the OVA peptide was always <2%, and % lysis of target (T) EG.7 cells expressing OVA by splenic effector (E) cells is shown in the table.

| E:T ratio | Naïve | FCA | Live BCG | BCG liposomes | *B. firmus* liposomes |
|---|---|---|---|---|---|
| 100:1 | 1 ± 1 | 17 ± 1.8 | 39 ± 2 | 30 ± 2 | 11 ± 0.6 |
| 33:1 | 2 ± 3 | 9 ± 1.7 | 23 ± 0.9 | 15 ± 1.5 | 9 ± 4 |
| 11:1 | 0 ± 1 | 3 ± 0.5 | 11 ± 3 | 8 ± 0.9 | 2.5 ± 0.3 |
| 3.7:1 | 1 ± 0 | 0.6 ± 0.7 | 4 ± 0.9 | 4 ± 0.4 | 1 ± 0.4 |

Table 5. Comparison of anti OVA antibody titres in sera of mice immunized with OVA in various adjuvant systems.

In all cases groups of 4 to 7 C57BL/6 mice were immunized with 15 μg OVA per injection at 0 and 3 weeks. BCG liposomes with OVA entrapped were prepared from 100% BCG TPL lipids mixed with DMPC/DMPG/cholesterol lipids from 0 to 100%, such that 0% BCG lipids were pure DMPC/DMPG/cholesterol liposomes. BCG OVA-free liposomes were separate injections of an equivalent amount of antigen-free BCG liposomes and OVA (unentrapped). For FCA OVA was mixed with FCA for the first injection and FIA for the second. Alum was Imject Alum to which OVA was bound. BCG live are BCG cells genetically modified to express OVA (see table 4 injection details). Blood was taken at various time points from first injection and anti OVA antibody in the sera was titrated by ELISA.

| Adjuvant | Day 10 | Day 31 | Day 41 |
|---|---|---|---|
| 100% BCG liposomes | 740 ± 380 | 23309 ± 16863 | 8266 ± 5240 |
| 50% BCG liposomes | 55 ± 54 | 14227 ± 4301 | 9643 ± 4287 |
| 10% BCG liposomes | 10 ± 0 | 1600 ± 1062 | 1499 ± 978 |
| 0% BCG liposomes | 10 ± 0 | 1065 ± 1020 | 857 ± 839 |
| BCG OVA-free liposomes | 0 | 388 ± 259 | 345 ± 350 |
| FCA | 5156 ±6722 | 41420 ± 26376 | 65671 ± 26080 |
| Alum | 472 ± 371 | 8306 ± 2074 | 12302 ± 6848 |
| BCG live | 50 | 0 | 0 |
| no adjuvant | 0 | 519 ± 552 | 821 ± 738 |

Table 6. Effect of antigen loading in BCG liposomes on induction of an immune response in mice.
6 wk pfi
A. CTL - % lysis of EG.7 targets

| E:T ratio | Naïve | 15 μg/1.8 mg | 15 μg/1.6 mg | 15 μg/0.22 mg |
|---|---|---|---|---|
| 100:1 | 0.9 ± 0.1 | 52 ± 2 | 51 ± 2 | 55 ± 2 |
| 33:1 | 0 ± 0.7 | 41 ± 3 | 36 ± 3 | 45 ± 3 |
| 11:1 | 0 ± 1 | 29 ± 3 | 22 ± 3 | 31 ± 5 |
| 3.7:1 | 0 ± 0.9 | 16 ± 2 | 10 ± 0.1 | 15 ± 2 |

B. Elispot – number of IFN secreting colonies

| OVA peptide | Naïve | 15 μg/1.8 mg | 15 μg/1.6 mg | 15 μg/0.22 mg |
|---|---|---|---|---|
| + | 0 | 23 ± 1 | 26 ± 3 | 24 ± 3 |
| - | 0 | 0 | 0 | 0 |

C. anti OVA antibody titres (2 to 4 mice/group)

| Mouse number | OVA, no adjuvant | 15 μg/1.8 mg | 15 μg/1.6 mg | 15 μg/0.22 mg |
|---|---|---|---|---|
| 1 | < 42 | 2922 | 2334 | 2965 |
| 2 | < 42 | 5552 | 1973 | 2346 |
| 3 | < 42 | - | 1692 | 3063 |
| 4 | < 42 | - | 3034 | 2797 |

**References**

Behr, M.A., B.G. Schroeder, J. N. Brinkman, R.A. Slayden, and C.E Barry (2000) A point mutation in the *mma3* gene is responsible for impaired methoxymycolic acid production in *Mycobacterium bovis* BCG strains obtained after 1927. J. Bacteriol. 182: 3394-3399.

Besra, G.S. and P.J. Brennan (1994) The glycolipids of mycobacteria. *In:* Mass spectrometry for characterization of microorganisms. American Chemical Society.

Bowersock, T.L. and S. Martin (1999) Vaccine delivery to animals. Advanced Drug Delivery Rev. 38: 167-194.

Chatterjee, D. and K.-H. Khoo (1998) Mycobacterial lipoarabinomannan: an extraordinary lipoheteroglycan with profound physiological effects. Glycobiology 8: 113-120.

Dudani, R. Chapedelaine, Y. van Fassen, H. Smith, D.K. Shen, H. Krishnna, L. Sad, S. (2002) Multiple mechanisms compensate to enhance tumor-protective CD8+ T cell response in the long-term despite poor CD8+ T cell priming initially: comparison between an acute versus a chronic intracellular bacterium expressing a model antigen. J. Immunol. 168: 5737-5745.

Fischer, W. and K. Leopold (1999) Polar lipids of four listeria species containing L-lysylcardiolipin, a novel lipid structure, and other unique phospholipids. International Journal of Systematic Bacteriology 49: 653-662.

Gilleron, M., C. Ronet, M. Mempel, B. Monsarrat, G. Gachelin, and G. Puzo (2001) Acylation state of the phosphatidylinositol mannosides from *Mycobacterium bovis* Bacillus Calmette Guérin and ability to induce granuloma and recruit natural killer T cells. J. Biol. Chem. 276: 34896-34904.

Kates, M (1986) Techniques of lipidology: isolation, analysis and identification of lipids. *In* Laboratory techniques in biochemistry and molecular biology. Vol. 3. Part 2. *Edited by* R.H. Burdon and P.H. van Knippenberg. Elsevier, New York.

Koike, Y., Y.C. Yoo, M. Mitobe, T. Oka, K. Okuma, S. Tono-oka, and I. Azuma (1998) Enhancing activity of mycobacterial cell-derived adjuvants on immunogenicity of recombinant human hepatitis B virus vaccine. Vaccine 16: 1982-1989.

Krishnan, L., C.J. Dicaire, G.B. Patel, and G.D. Sprott (2000) Archaeosome vaccine adjuvants induce strong humoral, cell-mediated, and memory responses: Comparison to conventional liposomes and alum. Infection and Immunity 68: 54-63.

Krishnan, L., S. Sad, G.B. Patel, and G.D. Sprott (2001) Archaeosomes induce long-term $CD8^+$ cytotoxic T cell response to entrapped soluble protein by the exogenous cytosolic pathway, in the absence of $CD4^+$ T cell help. J. Immunol. 165: 5177-5185

Leopold, K. and W. Fischer (1993) Molecular analysis of the lipoglycans of *Mycobacterium tuberculosis*. Analytical Biochemstry 208: 57-64.

Mára, M., J. Julák, E. Menčiková, J. Očenášková, and A. Dohnalová (1992) Effect of crude bacterial lipids on the course of *Listeria* infection in mice. Folia Microbiol. 37: 455-460.

Means, T.K., E. Lien, A. Yoshimura, S. Wang, D.T. Golenbock, and M.J. Fenton (1999) The CD14 ligands lipoarabinomannan and lipopolysaccharide differ in their requirement for Toll-like receptors. J. Immunol. 163: 6748-6755.

Mosmann, T.R., and T.A. Fong (1989) Specific assays for cytokine production by T cells. J. Immunol. Methods 116: 151-158.

Nigou, J., M. Gilleron, B. Cahuzac, J.-D. Bounéry, M. Herold, M. Thurnhert, and G. Puzo (1997) The phosphatidyl-myo-inositol anchor of the lipoarabinomannans from *Mycobacterium bovis* Bacillus Calmette Guérin. J. Biol. Chem. 272: 23094-23103.

Ortalo-Magné, A., A. Lemassu, M.-A. Lanéelle, F. Bardou, G. Silve, P. Gounon, G. Marchal, and M. Daffé (1996) Identification of the surface-exposed lipids on the cell envelopes of *Mycobacterium tuberculosis* and other mycobacterial species. J. Bacteriol. 178: 456-461.

Retzinger, G.S., S.C. Meredith, K. Takayama, R.L. Hunter, and F.J. Kézdy (1981) The role of surface in the biological activities of trehalose 6,6'-dimycolate. J. Biol. Chem. 256: 8208-8216.

Skinner, M.A., R. Prestidge, S. Yuan, T.J. Strabala, and P.L.J. Tan (2001) The ability of heat-killed *Mycobacterium vaccae* to stimulate a cytotoxic T-cell response to an unrelated protein is associated with a 65 kilodalton heat-shock protein. Immunology 102: 225-233.

Sprott, G.D., C.G. Choquet, and G.B. Patel (1995) Purification of ether lipids and liposome formation from polar lipid extracts of methanogenic bacteria. *In* F.T. Robb *et al.* (Eds.), Archaea: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 329-340.

Vijh, S. and Pamer, E.G. (1997) Immunodominant and subdominant CTL responses to *Listeria monocytogenes* infection. J. Immunol. 158: 3366-3371.

Wang, L., R.A. Slayden, C.E. Barry III, and J. Liu (2000) Cell wall structure of a mutant of *Mycobacterium smegmatis* defective in the biosynthesis of mycolic acids. J. Biol. Chem. 275: 7224-7229.

**CLAIMS:**

1. A liposome comprising a chloroform soluble and extractable total polar lipid of *Mycobacterium spp.*

2. A liposome comprising a chloroform soluble extractable total polar lipid of *Mycobacterium bovis* BCG.

3. A liposome according to claim 2, wherein the chloroform soluble and extractable polar lipid comprises at least one of phosphatidylinositol (PI), phosphatidylinositol mannoside ($PIM_1$), phosphatidylinositol dimannoside ($PIM_2$), mono and dipalmitoylated forms of $PIM_1$and $PIM_2$, acylated-phospholipids of 899, 1139 and 1155 m/z, phosphatidylethanolamine and cardiolipid.

4. A liposome according to claim 3, wherein the chloroform soluble and extractable polar lipid of *Mycobacterium bovis* BCG is in biologically pure form.

5. A liposome according to claim 4, wherein the chloroform soluble and extractable polar lipid is selected from the group consisting of PI, $PIM_1$, $PIM_2$, mono or dipalmitoylated forms of $PIM_1$ or $PIM_2$, acylated-phospholipids of 899, 1139 and 1155 m/z, and cardiolipid.

6. A liposome according to claim 5, wherein the chloroform soluble and extractable lipid is PI.

7. A liposome according to claim 5, wherein the chloroform soluble and extractable lipid is $PIM_1$.

8. A liposome according to claim 5, wherein the chloroform soluble and extractable lipid is $PIM_2$.

9. A liposome according to claim 5, wherein the chloroform soluble and extractable lipid is palmitoyl-$PIM_1$.

10. A liposome according to claim 5, wherein the chloroform soluble and extractable lipid is palmitoyl-$PIM_2$.

11. A liposome according to claim 4, wherein the chloroform soluble and extractable lipid is an acyl-phosphoglycerophosphate lipid of m/z 899, 1139 or 1155 comprising two sn-1,2 fatty acyl chains of tuberculosteric acid (C19:0), or a first chain is tuberculosteric acid and a second chain is palmitic acid (C16:0).

12. A liposome according to any one of claims 1 to 11, wherein the chloroform soluble and extractable polar lipid is obtainable by a hot 50% ethanol extraction.

13. A liposome comprising an isolated lipid fraction in biologically pure form from total polar lipids of *Mycobacterium bovis* BCG and an associated antigen.

14. A liposome according to any one of claims 1 to 13, wherein the lipid ingredient is synthesized chemically to correspond to the structure of a lipid isolated in biologically pure form from a mycobacterium.

15. A liposome according to claim 5, additionally comprising lipid phosphatidylethanolamine in biologically pure form.

16. A liposome according to claim 2, comprising the chloroform soluble and extractable polar lipid of *Mycobacterium bovis* BCG, and other lipid.

17. A liposome according to claim 16, wherein the other lipid is selected from the group consisting of phosphatidylcholine, phosphatidylglycerol, cholesterol and a mixture thereof.

18. A liposome according to any one of claims 1 to 17, wherein said liposome is multilamellar.

19. A liposome according to any one of claims 1 to 17, wherein said liposome is unilamellar.

20. A liposome vaccine composition comprising a liposome according to claim 2, wherein the liposome contains an associated antigen.

21. A liposome vaccine composition comprising a liposome according to any one of claims 1 to 12 and 14 to 17, wherein the liposome contains an associated antigen.

22. A liposome vaccine composition according to claim 20 or 21, wherein the antigen is a protein.

23. A method for preparing a liposome according to any one of claims 1 to 19 which method comprises drying chloroform soluble and extractable lipid and then hydrating said dried lipid at a temperature of 65 to 75°C in water or phosphate buffered saline (PBS).

24. A method according to claim 23, wherein said temperature is 65°C.

25. A method according to claim 23, wherein said liposome resulting from said method is multilamellar.

26. A method according to claim 25, additionally comprising reducing the size of a multilamellar liposome at a temperature of 65°C to yield a unilamellar liposome.

27. A method according to claim 25, wherein an antigen is entrapped in said multilamellar liposome by inclusion of said antigen in water or phosphate buffered saline.

28. A method according to claim 26, wherein an antigen is entrapped in said unilamellar liposome by inclusion of said antigen in water or phosphate buffered saline.

29. Use of a liposome according to any one of claims 1 to 11 to activate dendritic cells to secrete cytokines and modulate an immune response in a mammal.

30. The use of a liposome according to any one of claims 2, 6 and 11, to activate dendritic cells wherein activation results in uptake of dimethylthioazol diphenyltetrazolium bromide (MTT) by said dendritic cells.

31. The use of a liposome according to any one of claims 7 to 11, to activate dendritic cells wherein activation elicits secretion of Interleukin-12 (IL-12) by said dendritic cells.

32. The use of a liposome according to claim 6, to activate dendritic cells wherein activation elicits secretion of tumour necrosis factor (TNF) by said dendritic cells.

33. The use of a liposome according to claim 2, to activate dendritic cells wherein activation elicits secretion of Interleukin-12 (IL-12) and tumour necrosis factor (TNF) by said dendritic cells.

34. The use of a liposome according to any one of claims 29 to 33 wherein said liposome additionally comprises an antigen.

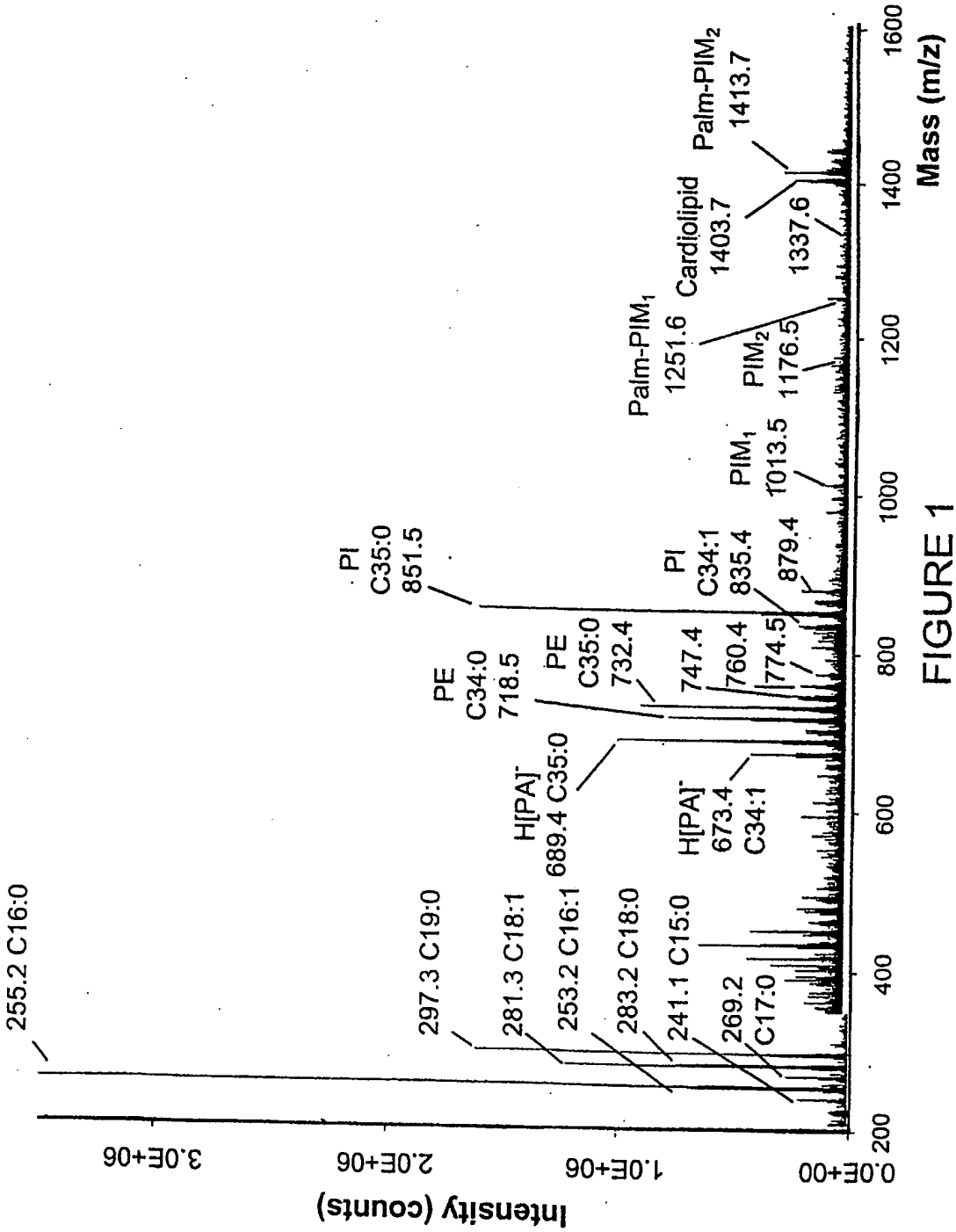
35. Use of a liposome vaccine composition according to claim 20 to elicit a strong immune response in a mammal.

36. The use according to claim 35, wherein the liposome serves both as a carrier for the antigen and as a modulator of an immune response.

37. The use according to claim 35, wherein the immune response is an antigen specific antibody response and an antigen specific cytotoxic T cell response.

38. Use of a vaccine composition according to claim 20 to elicit protection in an animal against cancer.

39. Use of a liposome according to claim 20 to direct an immune response to confer protection against a pathogen or a cancer.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600
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1000
1200
1400
1600
Mass (m/z)
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297.3 C19:0
281.3 C18:1
253.2 C16:1
283.2 C18:0
241.1 C15:0
269.2
C17:0
H[PA]⁻
689.4 C35:0
H[PA]⁻
673.4
C34:1
PE
C34:0
718.5
PE
C35:0
732.4
PI
C35:0
851.5
747.4
760.4
774.5
PI
C34:1
835.4
879.4
PIM1
1013.5
Palm-PIM1
1251.6
PIM2
1176.5
Cardiolipid
1403.7
1337.6
Palm-PIM2
1413.7

FIGURE 1

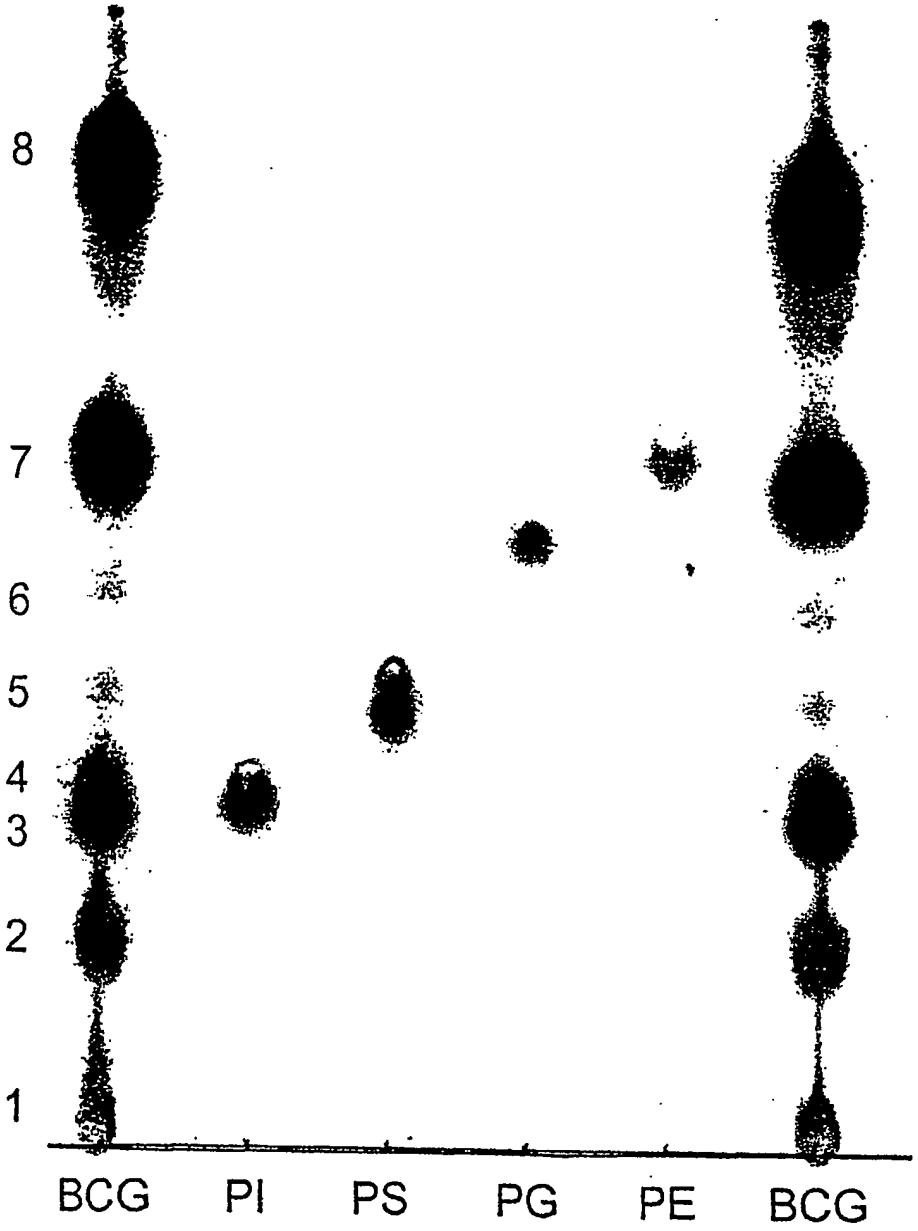
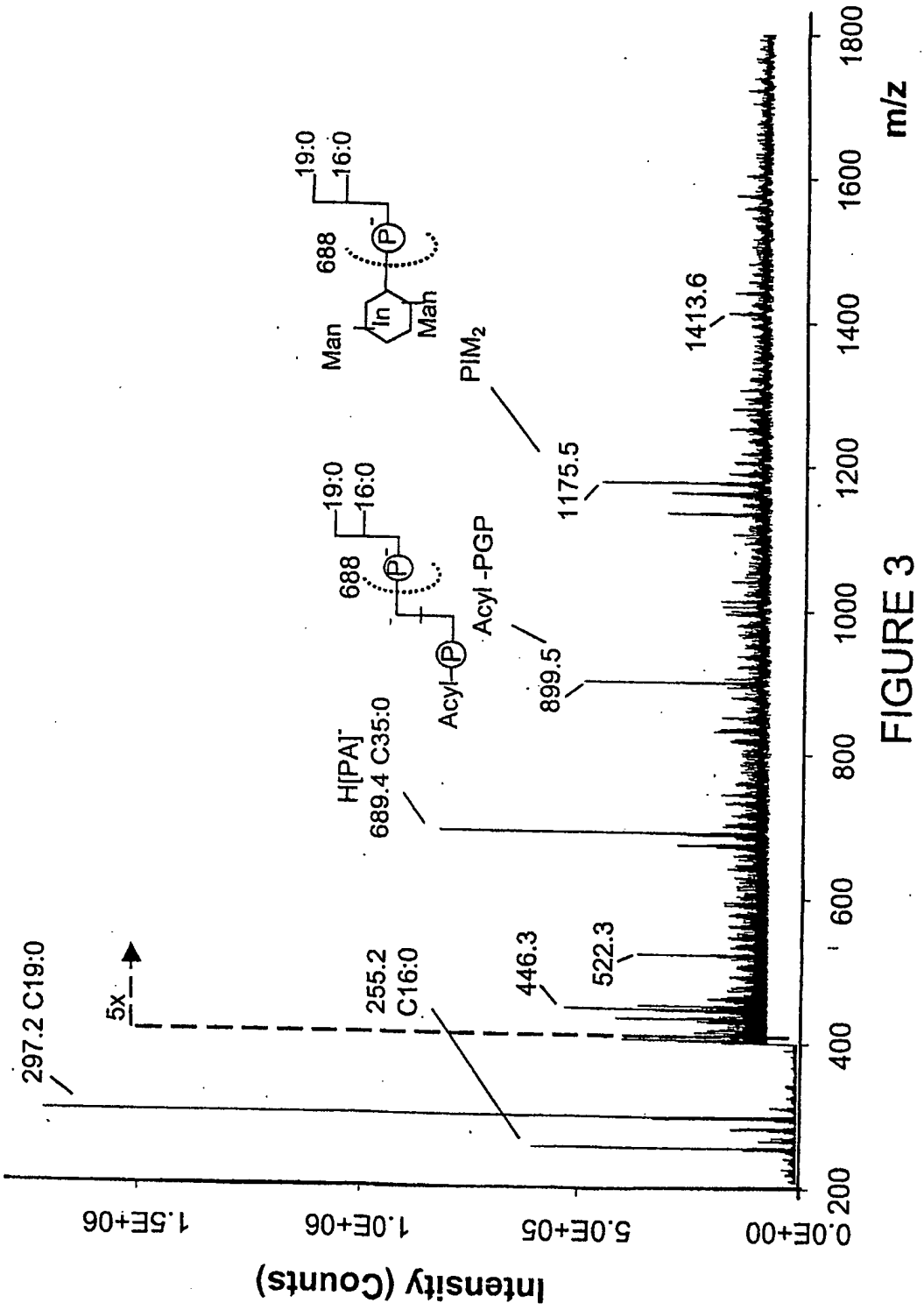



FIGURE 2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1.0E+06
1.5E+06
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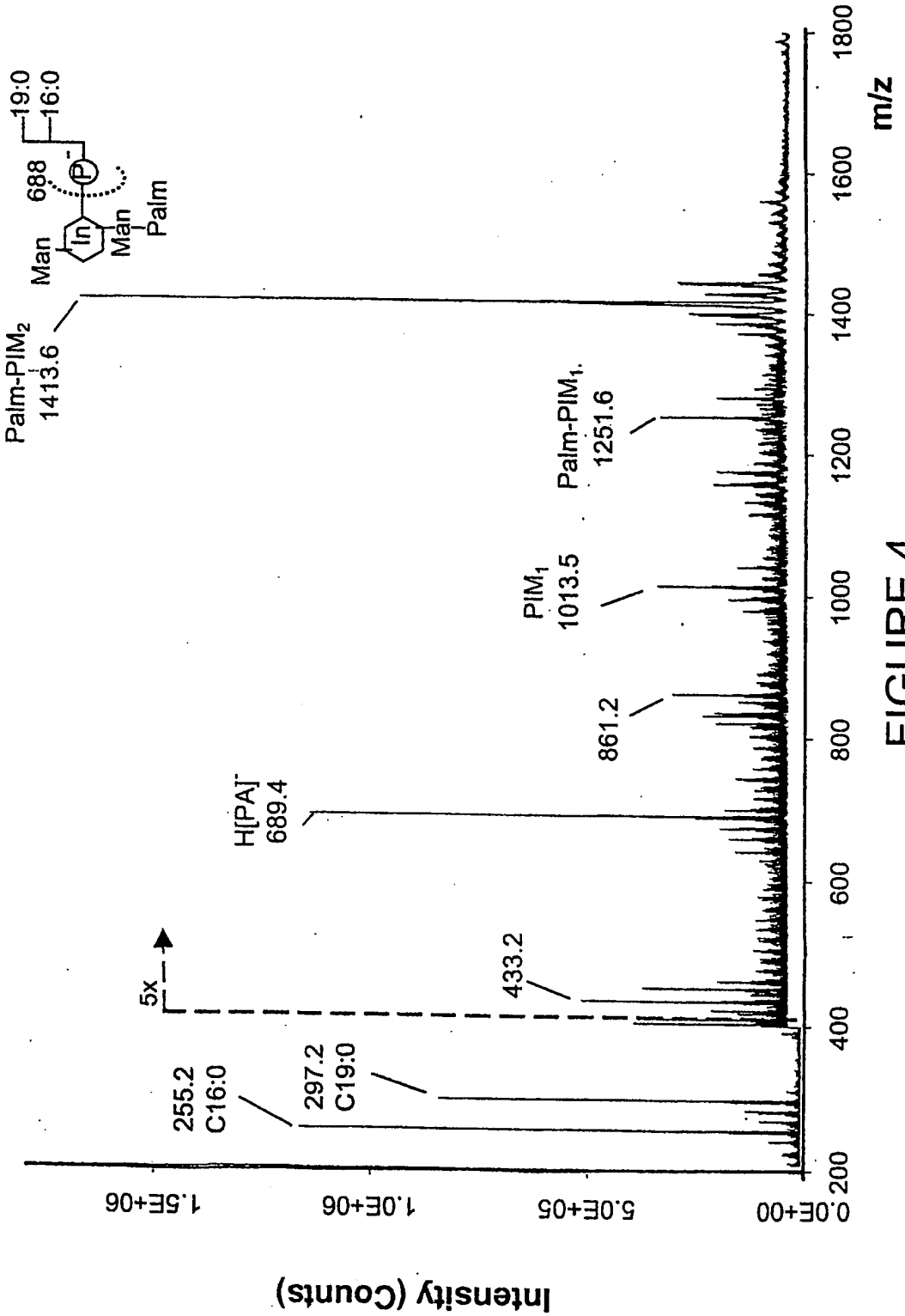
400
600
800
1000
1200
1400
1600
1800
m/z
297.2 C19:0
5x
255.2 C16:0
446.3
522.3
H[PA]⁻ 689.4 C35:0
Acyl-P
688
19:0
16:0
Acyl -PGP
899.5
1175.5
PIM2
Man
In
Mah
1413.6

FIGURE 3


Intensity (Counts)
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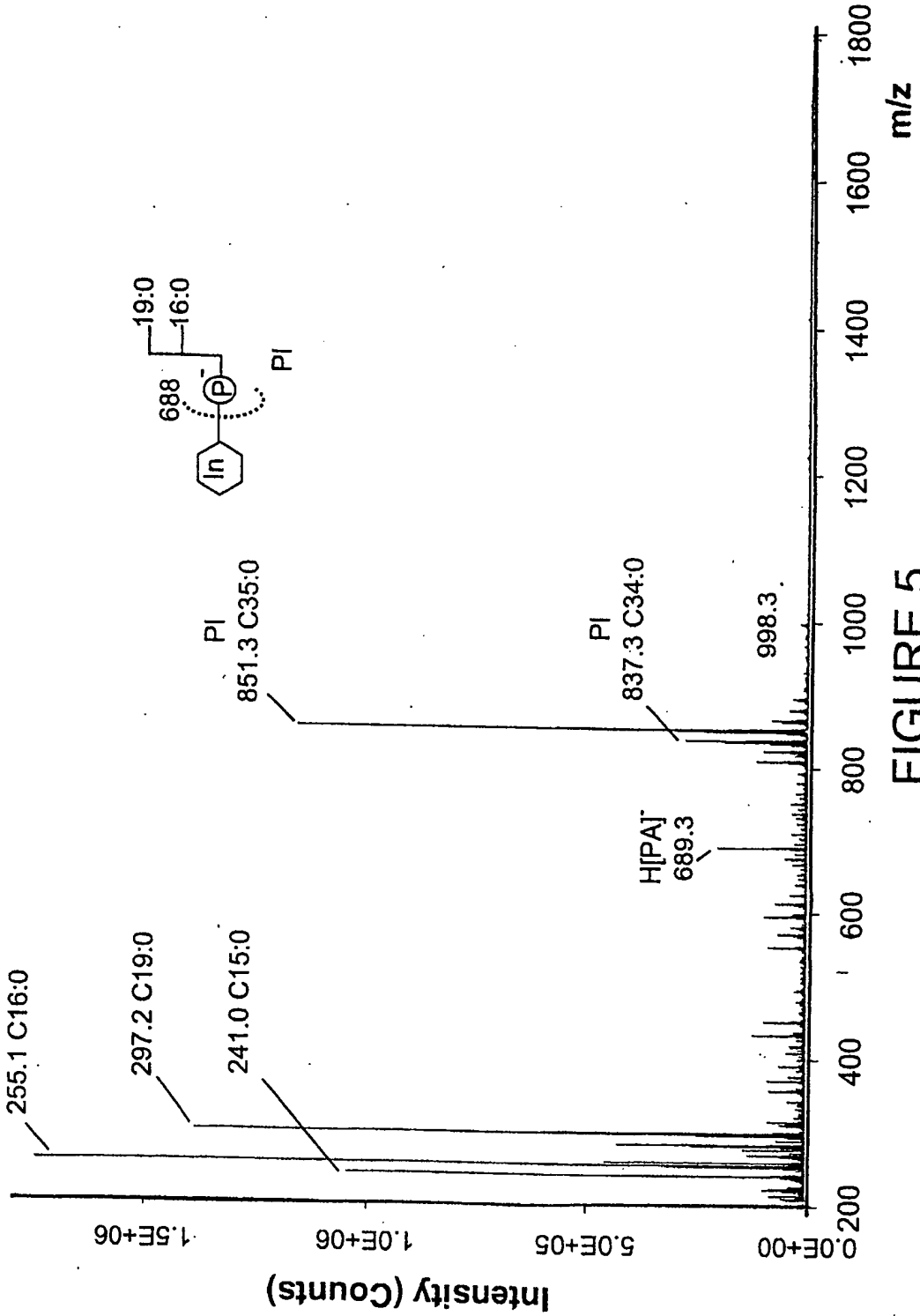
1.0E+06
1.5E+06
200
400
600
800
1000
1200
1400
1600
1800
m/z
5x
255.2
C16:0
297.2
C19:0
433.2
H[PA]⁻
689.4
861.2
PIM1
1013.5
Palm-PIM1.
1251.6
Palm-PIM2
1413.6
Man
In
Man
Palm
688
19:0
16:0

FIGURE 4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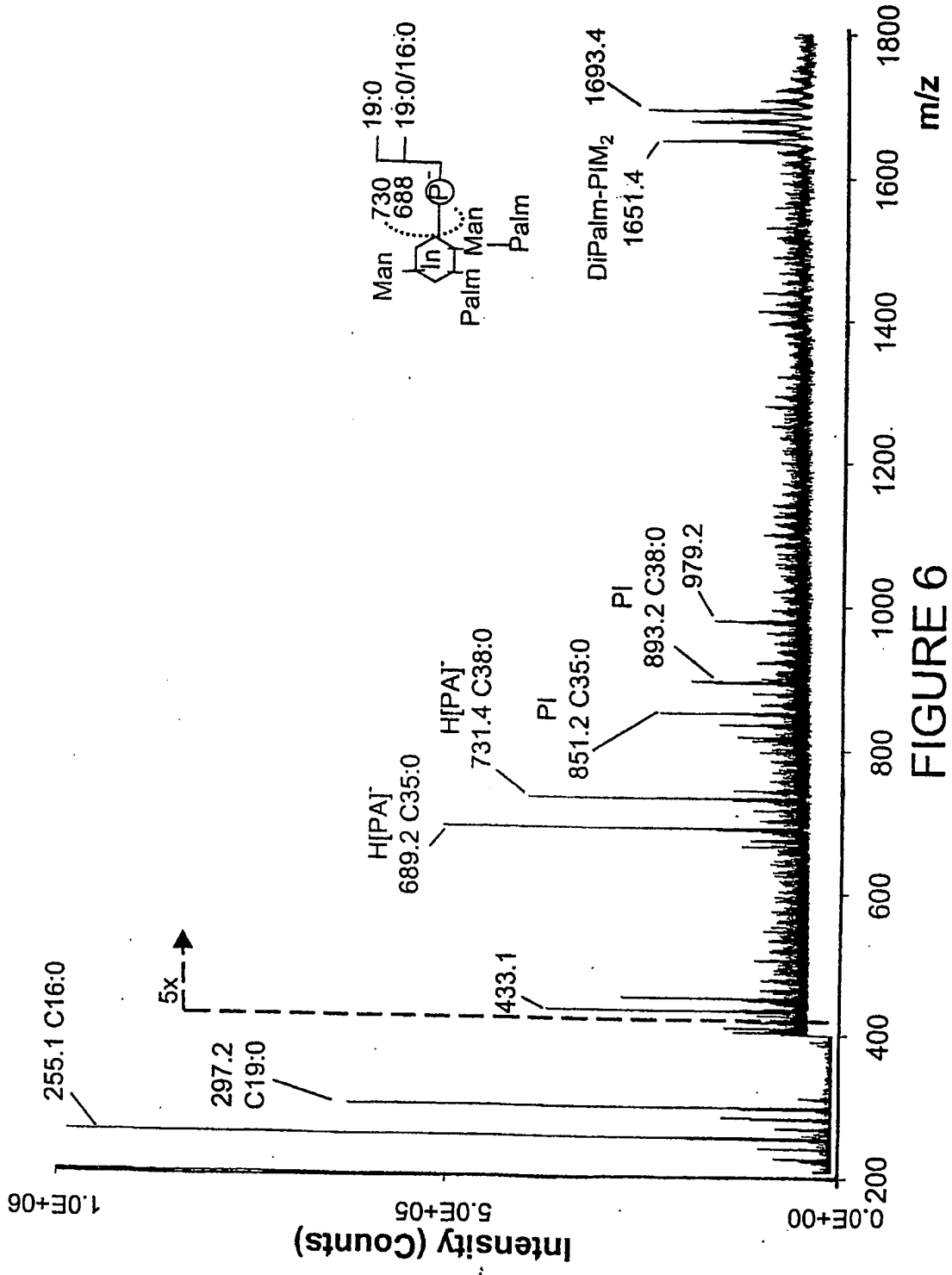
1.0E+06
1.5E+06
200
400
600
800
1000
1200
1400
1600
1800
m/z
255.1 C16:0
297.2 C19:0
241.0 C15:0
H[PA]-
689.3
PI
851.3 C35:0
PI
837.3 C34:0
998.3
19:0
16:0
688
PI
In

FIGURE 5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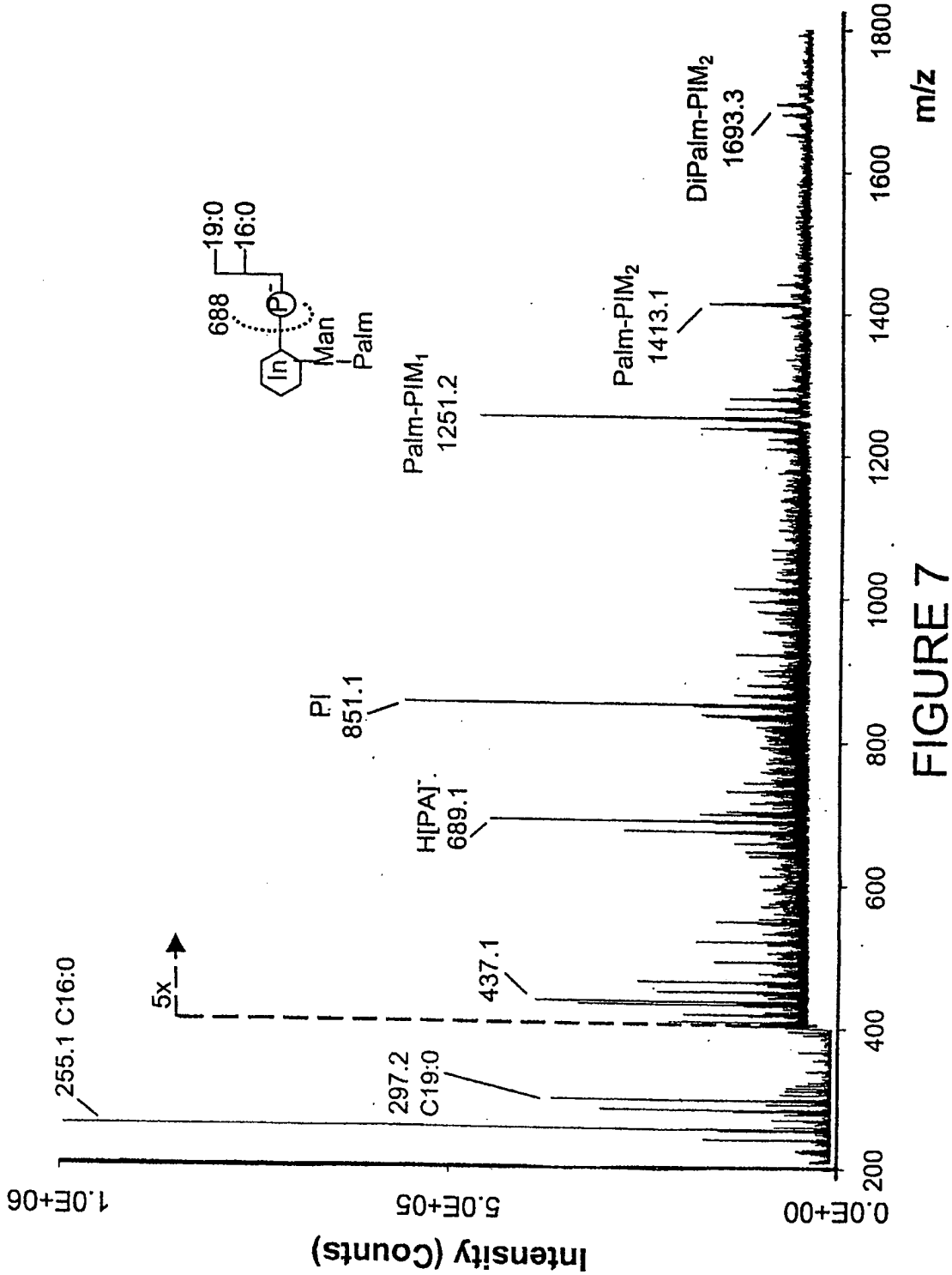
0.0E+00
200
400
600
800
1000
1200
1400
1600
1800
m/z
255.1 C16:0
297.2 C19:0
5x
433.1
H[PA]⁻ 689.2 C35:0
H[PA]⁻ 731.4 C38:0
PI 851.2 C35:0
PI 893.2 C38:0
979.2
DiPalm-PIM2 1651.4
1693.4
Man
730
688
19:0
19:0/16:0
In
P
Palm
Man
Palm

FIGURE 6


Intensity (Counts)
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1.0E+06
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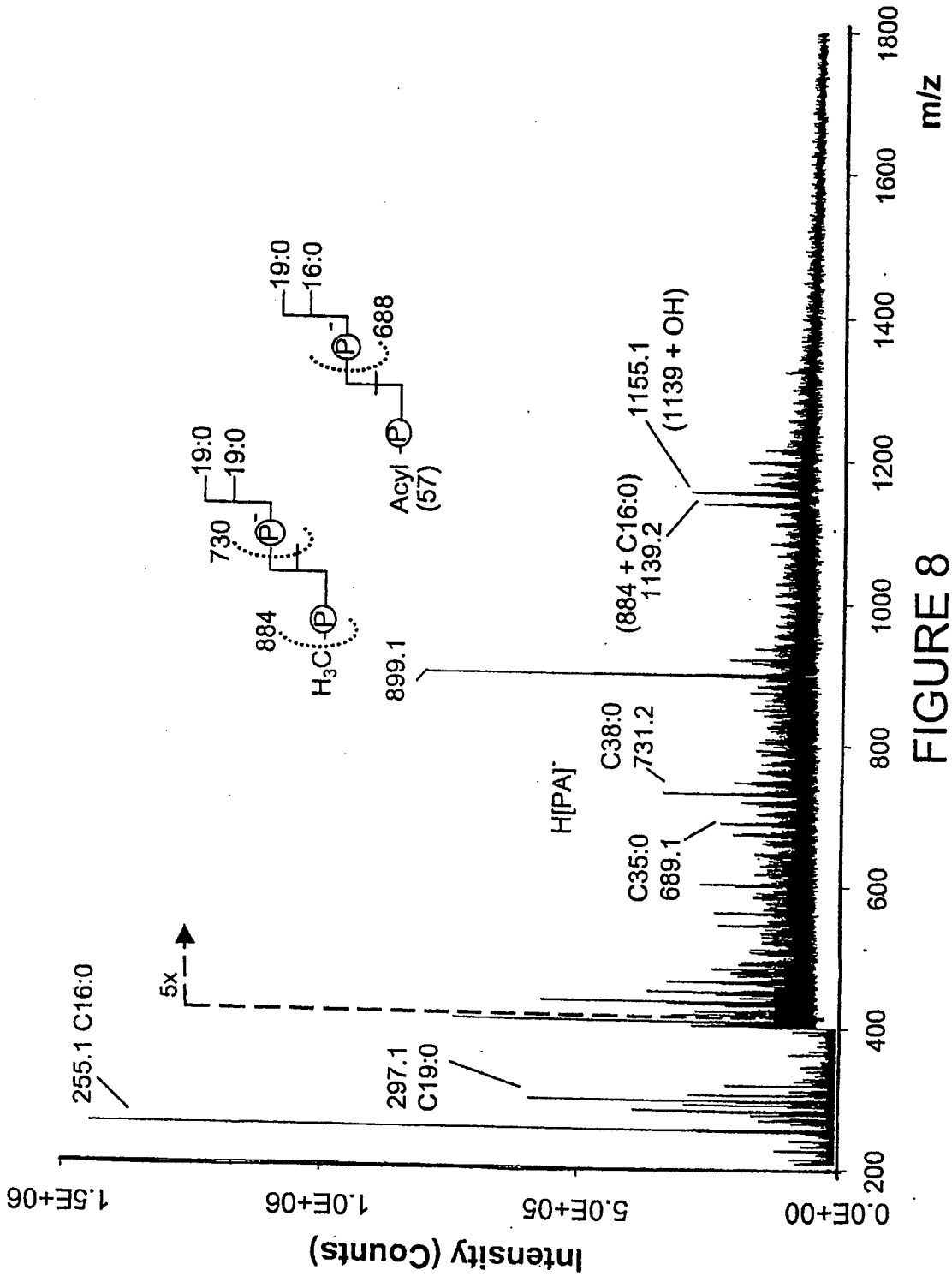
400
600
800
1000
1200
1400
1600
1800
m/z
255.1 C16:0
297.2 C19:0
437.1
5x
H[PA]⁻ 689.1
PI 851.1
Palm-PIM1 1251.2
Palm-PIM2 1413.1
DiPalm-PIM2 1693.3
688
19:0
16:0
In
Man
Palm

FIGURE 7


Intensity (Counts)
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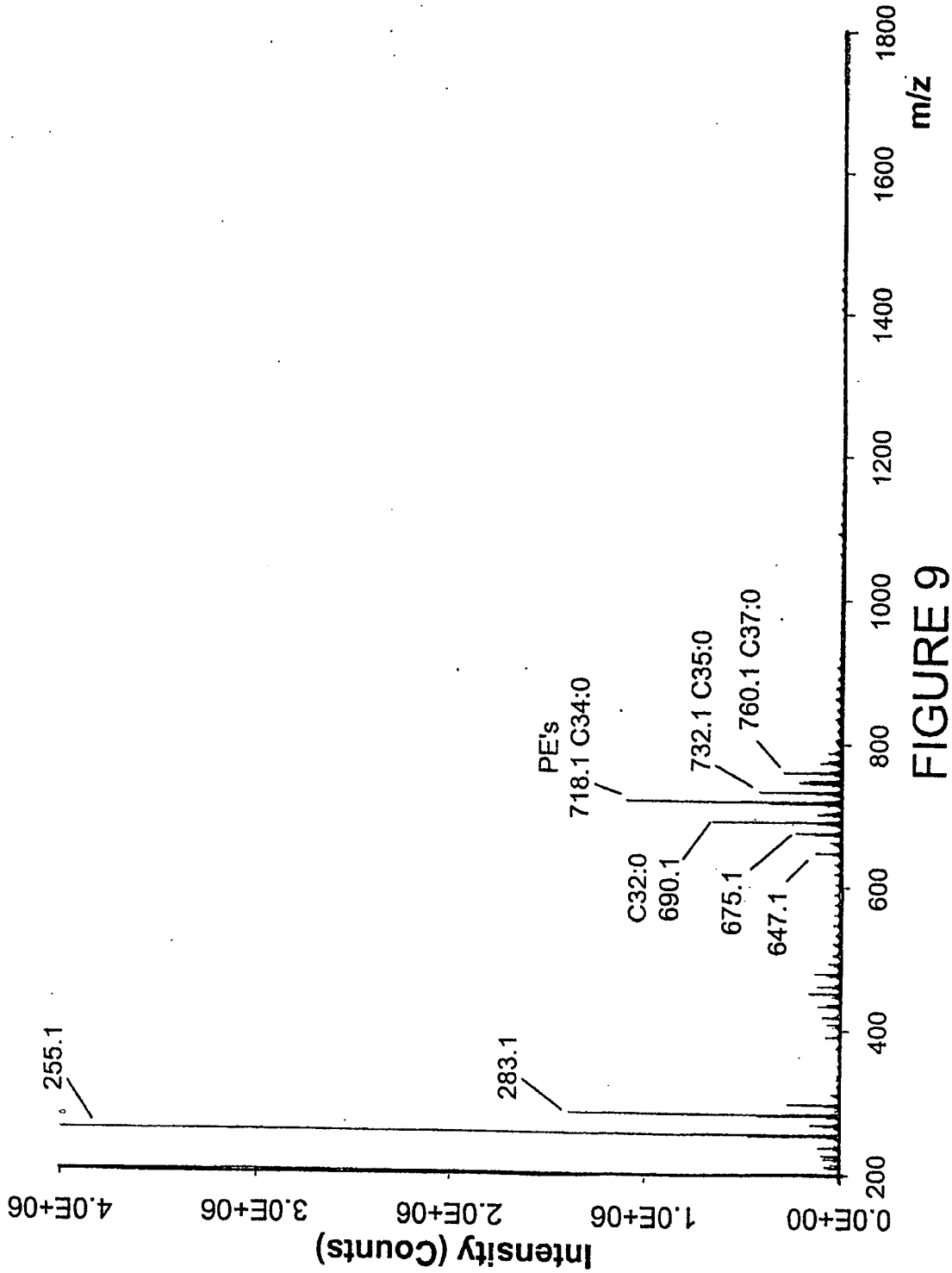
1.0E+06
1.5E+06
m/z
200
400
600
800
1000
1200
1400
1600
1800
255.1 C16:0
5x
297.1 C19:0
C35:0 689.1
H[PA]-
C38:0 731.2
899.1
884
730
19:0
19:0
H3C
Acyl (57)
688
19:0
16:0
(884 + C16:0) 1139.2
1155.1 (1139 + OH)

FIGURE 8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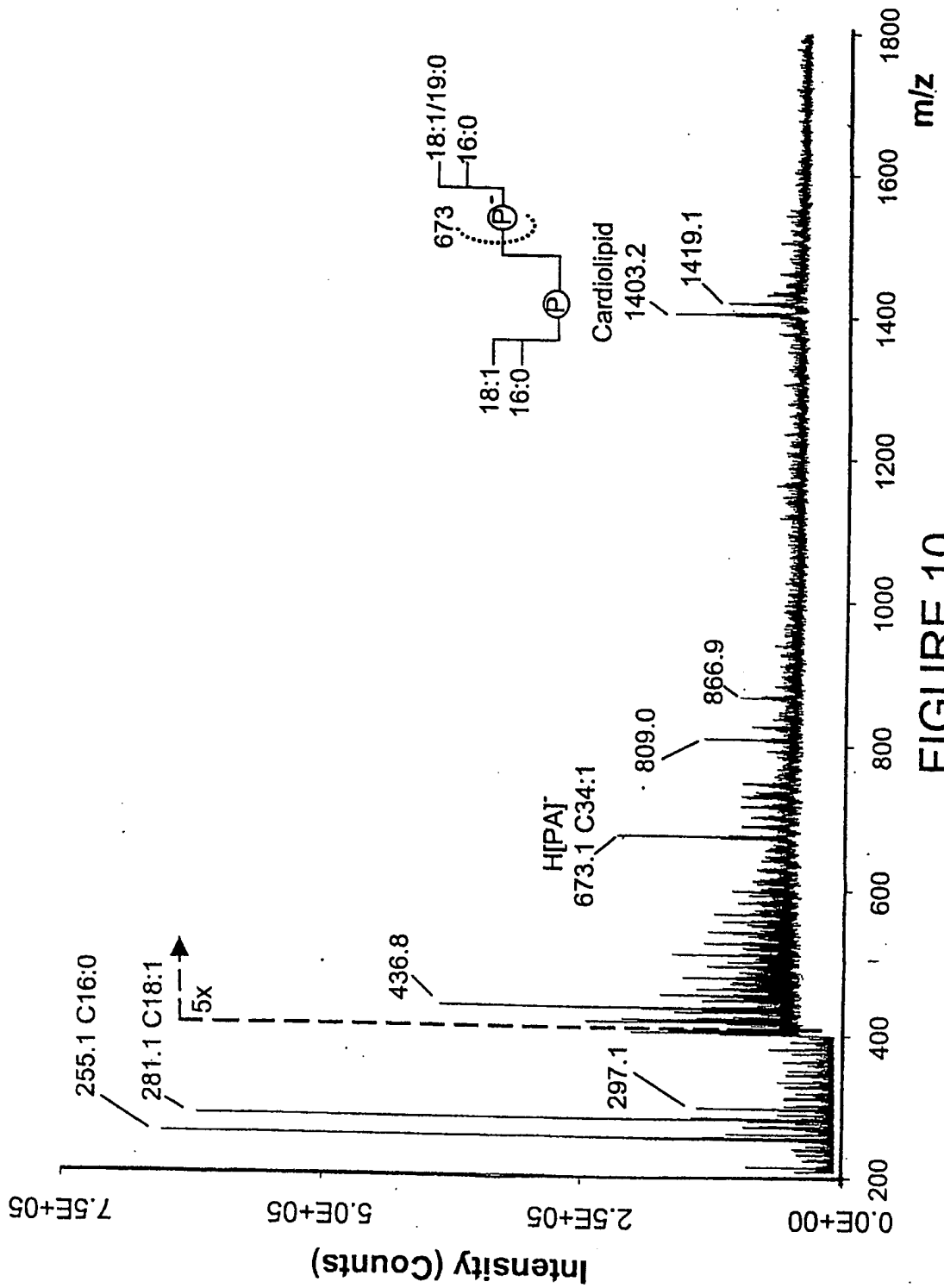
400
600
800
1000
1200
1400
1600
1800
m/z
255.1
283.1
PE's
718.1 C34:0
732.1 C35:0
760.1 C37:0
C32:0
690.1
675.1
647.1

FIGURE 9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7.5E+05
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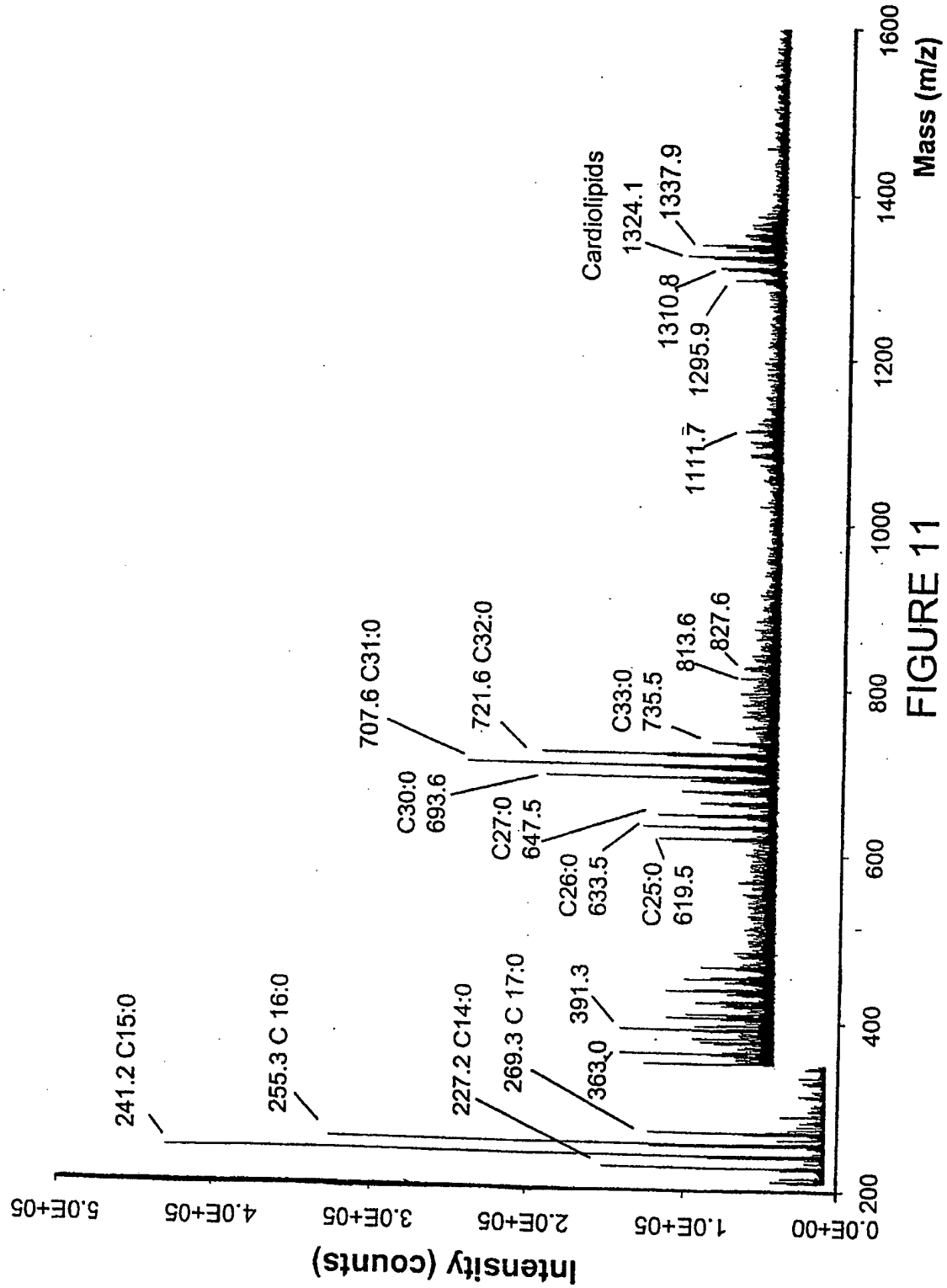
400
600
800
1000
1200
1400
1600
1800
m/z
255.1 C16:0
281.1 C18:1
5x
436.8
297.1
H[PA]-
673.1 C34:1
809.0
866.9
673
18:1/19:0
16:0
18:1
16:0
Cardiolipid
1403.2
1419.1

FIGURE 10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5.0E+05
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400
600
800
1000
1200
1400
1600
Mass (m/z)
241.2 C15:0
255.3 C 16:0
227.2 C14:0
269.3 C 17:0
391.3
363.0
C26:0
633.5
C25:0
619.5
C27:0
647.5
C30:0
693.6
707.6 C31:0
721.6 C32:0
C33:0
735.5
813.6
827.6
1111.7
1295.9
1310.8
Cardiolipids
1324.1
1337.9

FIGURE 11

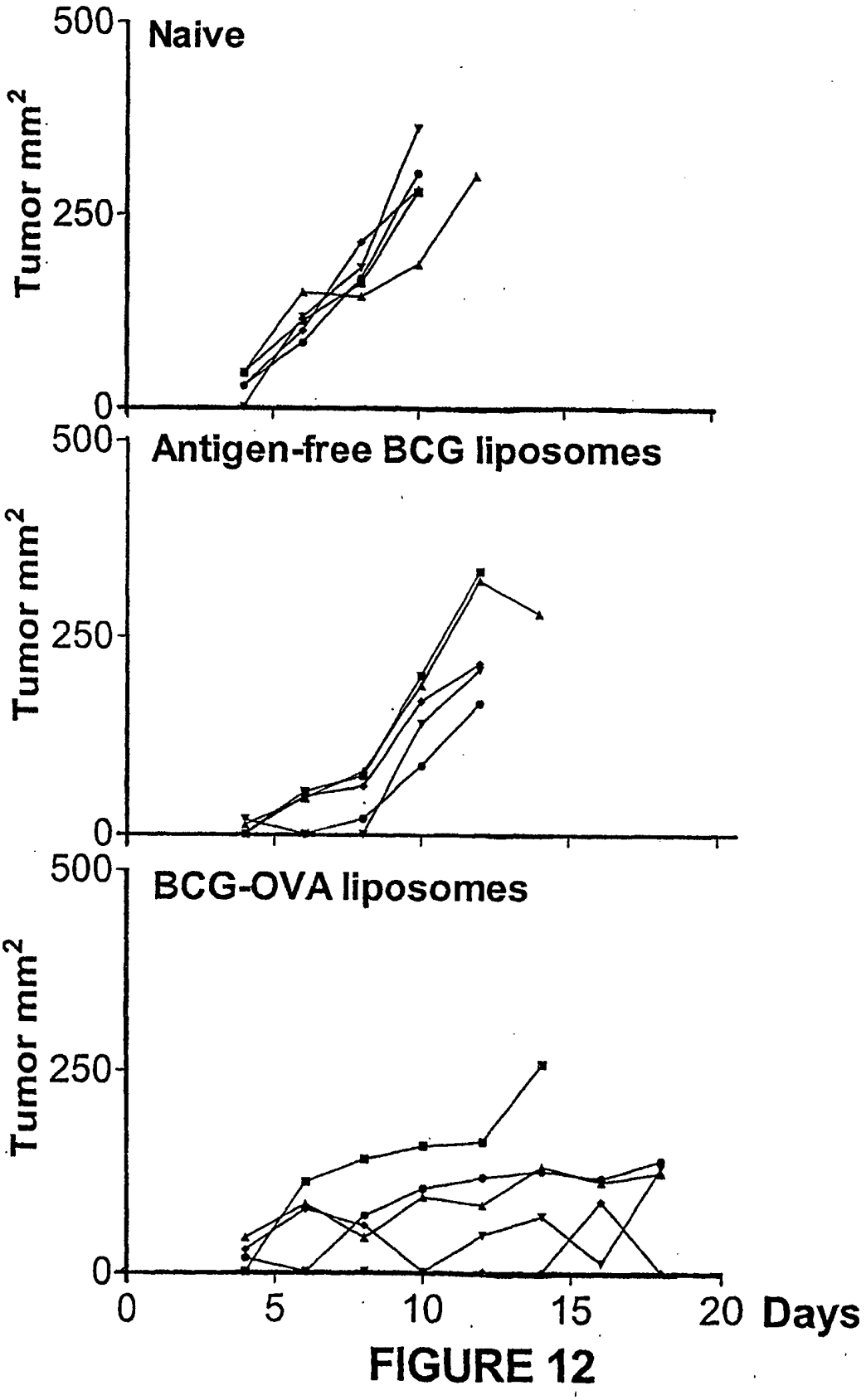
Naive
Antigen-free BCG liposomes
BCG-OVA liposomes
Tumor mm2
500
250
0
0
5
10
15
20
Days

FIGURE 12